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Neuro-Signals

Invertebrate Neural Networks

Editors
Yung Hou Wong, Hong Kong
Joseph T.Y. Wong, Hong Kong

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Editorial

Neural networking forms the basis of learning and memory, which in turn are the foundation of intelligence. As early as the 1970s, studies in invertebrate systems revealed that structural changes at synapses are related to learning and memory storage. Invertebrate models not only provide simple systems for the studies of complex behavior, many systems are also amenable for genetic studies. While neural networking is now synonymous with computational approaches, we have yet to explore the full potential of what invertebrate neuronal systems can provide. With the advent of genomics and proteomics, it is now pertinent to have a fresh look at some of the invertebrate systems. We have gathered reviews across a wide spectrum of invertebrate systems. The cnidarians consist of organisms capable of behavior generated from simple neural net, or from centralized system, as in the case of the jellyfish. Crustaceans and insects have been useful models of understanding rhythmic behavior. Synaptic plasticity, in relation to memory, was first discovered in Alphysia. Cephalopods are well known for their capacity of intelligence behavior. *Drosophila*, with the advantage of genetics, is useful for the molecular study of network guidance and formation.

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Review

NEURO-SIGNALS

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Central Neural Circuitry in the Jellyfish *Aglantha*

A Model 'Simple Nervous System'

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Key Words

Cnidaria · Medusa · Hydromedusa · Nerve net · Hair cell · Giant axon · Epithelial conduction · FMRFamide · Neural network · Escape behavior

Abstract

Like other hydrozoan medusae, *Aglantha* lacks a brain, but the two marginal nerve rings function together as a central nervous system. Twelve neuronal and two excitable epithelial conduction systems are described and their interactions summarized. *Aglantha* differs from most medusae in having giant axons. It can swim and contract its tentacles in two distinct ways (escape and slow). Escape responses are mediated primarily by giant axons but conventional interneurons are also involved in transmission of information within the nerve rings during one form of escape behavior. Surprisingly, giant axons provide the motor pathway to the swim muscles in both escape and slow swimming. This is possible because these axons can conduct calcium spikes as well as sodium spikes and do so on an either/or basis without overlap. The synaptic and ionic bases for these responses are reviewed. During feeding, the manubrium performs highly accurate flexions to points at the margin. At the same time, the oral lips flare open. The directional flexions are conducted by FMRFamide immunoreactive nerves, the lip flaring by an excitable epithelium lining the radial canals. Inhibition of swimming during feeding is due to impulses propagated centrifugally in the same epithelium. *Aglantha* probably evolved from an ancestor possessing a relatively simple wiring plan, as seen in other hydromedusae. Acquisition of giant axons resulted in considerable modification of this basic plan, and required novel solutions to the problems of integrating escape with non-escape circuitry.

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Introduction

The term 'central nervous system' can legitimately be applied to hydromedusan nervous systems as these animals have concentrations of hundreds of axons running in parallel forming 'nerve rings' in the margin. There are two such rings, an inner and an outer (fig. 1C), but axonal processes cross between them at many points and the two rings essentially function as single unit. In cross sections of the nerve rings of *Aglantha*, a total of about 800 axon profiles are seen, most of them less than 1 μm in diameter. As in medusae generally [1] they lack glial sheaths, although bundles of axons are sometimes partially separated by epithelial processes. In all species investigated the nerve rings include several functionally distinct nerve

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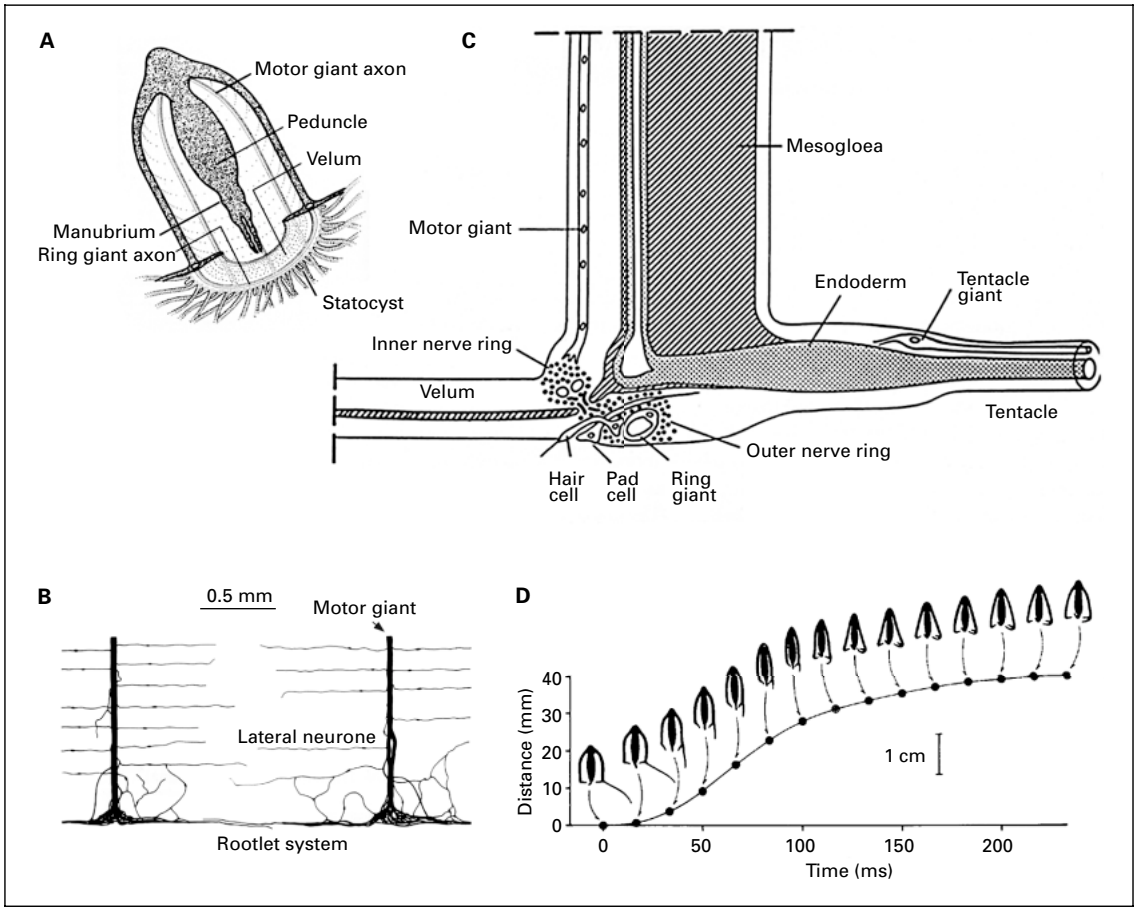

Fig. 1. Anatomy. **A** *Aglantha* cut in half vertically [15]. **B** Schematic representation of neighboring motor giant axons injected with Lucifer yellow. The dye has penetrated the lateral neurons and the system of rootlet interneurons [48]. **C** Perradial section through the margin. There are about 800 neurons in a typical cross section through the nerve rings, most of them less than 1.0 μm in diameter. Nerves cross the mesogloea between the inner and outer rings. The giant axons are conspicuously larger and both the ring and tentacle giants have prominent central vacuoles. The 'pad cell' is an enigmatic structure whose function is still not understood [57]. **D** Profiles of *Aglantha* during a single escape swimming contraction, showing distance travelled over time [15].

pathways, and they often interact in complex ways. The fact that the central nervous system takes the form of an annulus rather than a single, compact ganglion does not make it any less 'central' in terms of the functions carried on within it. The annular configuration is simply an adaptation to radial symmetry [2, 3]. It does mean, however, that pacemakers [4, 5] and synaptic interactions are replicated at numerous points around the ring, rather than being localized to specific zones as in the neuropil of a conventional ganglion. A practical advantage of the annular shape of the central nervous system is that a small piece of the margin containing part of the nerve ring can be cut out and pinned out for electrophysiology and it will have all the same systems and show the same synaptic interactions as the intact animal.

Hydromedusae differ in fundamental respects from jellyfish in the classes Scyphozoa and Cubozoa and no attempt will be made here to draw comparisons with members of these groups or to cover the extensive literature dealing with them. Fortunately, Satterlie's landmark review [6] covers nervous organization in all three classes, showing very clearly how they resemble one another and how they differ. Of the Hydromedusae, two species have been studied in most depth, *Polyorchis penicillatus* and *Aglantha digitale.* The focus here will be on *Aglantha*, where 14 physiologically distinct systems have been identified [7] but the *Polyorchis* work (summarized in [2, 8, 9]) offers instructive parallels and will be referred to frequently, along with work on several other species.

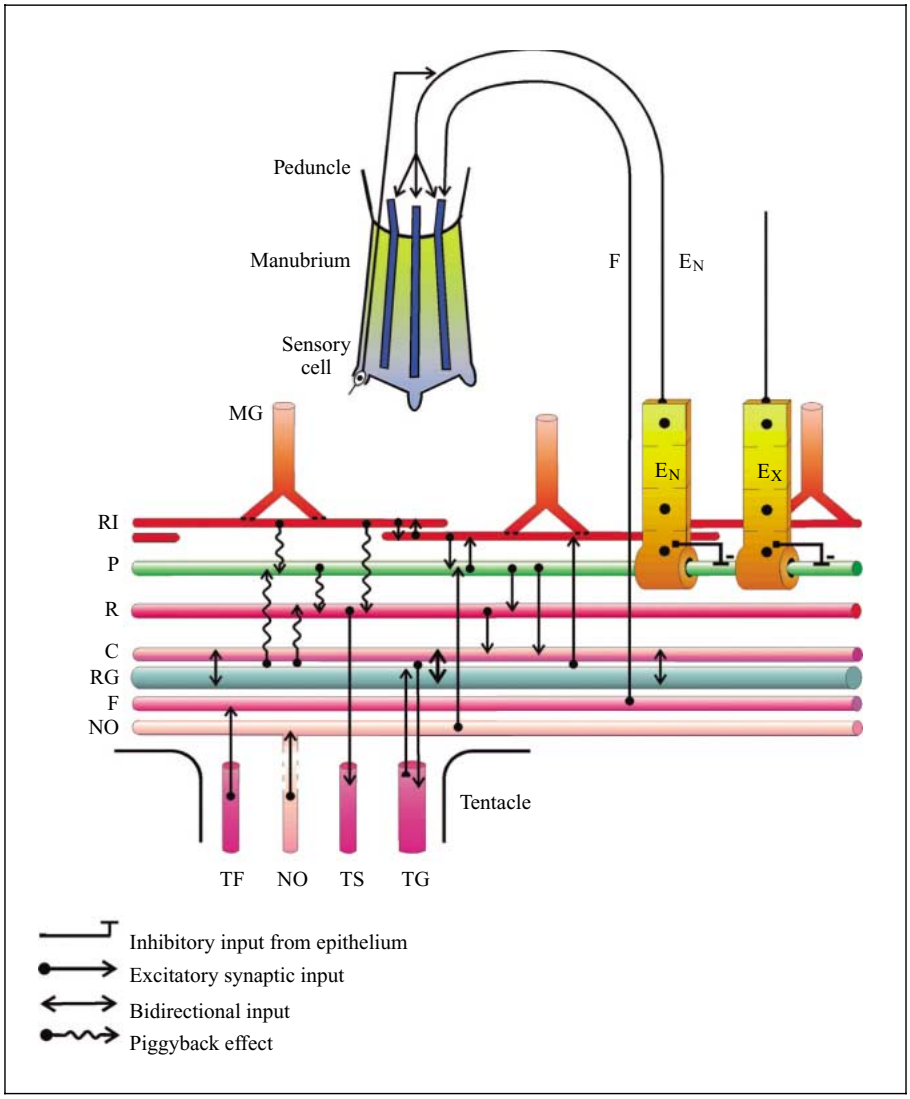

Fig. 2. Circuitry. The principal pathways involved in locomotion, the control of tentacle contractions and food manipulation. Three of the eight longitudinal muscle bands lying in the wall of the manubrium are shown. Gap junctions are indicated by incomplete partitions between cells [39]. C = Carrier system; E_n = endodermal epithelial pathway; E_x = exumbrellar, ectodermal epithelial pathway; F = flexion system used in pointing behaviour; MG = motor giant axon; NO = nitric oxide pathway; P = pacemaker system; R = relay system; ring giant = ring giant axon; RI = rootlet interneurons; TF = the portion of the F system that originates in the tentacles; TG = tentacle giant axon; TS = slowly conducting tentacle system.

Aglantha digitale is a hydrozoan medusa in the family Rhopalonematidae. It is a small transparent jellyfish 1–2 cm long with numerous tentacles extending from around the lower margin (fig. 1A). Members of this family differ from typical hydromedusae in being pelagic throughout their entire life cycle, with no settled hydroid stage. Though sometimes brought to the surface by mixing or upwelling, *Aglantha* typically inhabits the mesopelagic realm of the sea. In the waters around Vancouver Island it lives at 50–200 m [10, 11], but is also often found at the surface during the spring at the Friday Harbor Laboratories of the University of Washington, USA, where most of the work covered here was done.

References to *Aglantha*'s nervous organization go back to the 19th century [12], but modern interest in the topic stems from (a) the observation that this jellyfish can swim in two distinct ways [13], and (b) the discovery of giant axons ('motor giants', fig. 1B, C) associated with the swim musculature [14]. The role of the giant axons in mediating one of the two forms of swimming (escape swimming, fig. 1D) was soon established [15, 16], but it has taken another 23 years for anything like a complete picture of the main circuitry to emerge [39] and many puzzles remain. I have tried here to draw attention to some of the major lacunae.

A problem facing *Aglantha* workers is the animal's small size and the difficulty of obtaining intracellular recordings from its neurons. Our analysis has depended heavily on extracellular recordings. This disadvantage is offset by the relative ease with which microelectrode

recordings can be obtained from the swim muscles and from the giant axons. By studying the synaptic inputs into the giant axons while recording extracellularly from the nerve rings it has been possible to relate different input events to particular neural sub-systems and to build up a fairly comprehensive picture of the wiring (fig. 2). The reader may find it useful to refer to this figure frequently in the following pages even where it is not specifically cited, as it summarizes the whole story.

The present review covers the circuitry underlying the two sorts of swimming and associated tentacular contractions and summarizes recent findings on the pathways mediating feeding behaviour.

Swimming

There are two sorts of swimming, 'slow' and 'escape', and both employ the same effectors, the subumbrellar swim muscles. Slow swimming resembles the swimming of other hydromedusae, but escape swimming is unique to *Aglantha* and its relatives. The swim muscles lie in the ectoderm and are composed of myoepithelial cells whose striated, contractile processes run circularly forming a continuous sheet [14]. The cells are electrically coupled and current injected at one point depolarizes adjacent cells [18], but the spread is strictly local and propagated spikes have not been observed. This is contrary to the situation in most hydromedusae and siphonophores where myoid conduction is the norm, and the swimming motor neurons are, with a few exceptions [20], confined to the margin [2]. In *Aglantha*, excitation is spread across the muscle sheet by nerves, allowing the muscles to be excited in different ways during slow and escape swimming.

Both sorts of swimming are accompanied by contractions of the tentacles, but again these contractions are brought about in two different ways (see page 13).

Slow Swimming

Like other hydromedusae, *Aglantha* performs slow, rhythmic swimming when moving around normally. These contractions are generated endogenously by pacemaker neurons located in the inner marginal nerve ring (fig. 3A) and are exhibited in bursts of variable duration, sometimes at fairly regular intervals [21]. In certain, larger medusae (e.g. *Polyorchis*) it has been possible to record intracellularly from the equivalent units and to inject dyes [22, 23], but this has not been achieved in *Aglantha*, and we do not know how many neurons are involved or whether they are electrically coupled as in *Polyorchis* [23, 24]. In *Aglantha* they conduct circularly around the margin at velocities of $<0.5\ m \cdot s^{-1}$ [7], however, and presumably include some fairly large units.

Excitation spreads up the subumbrella from the margin rather slowly during slow swimming and the contractions evoked in the swim muscles are relatively weak, each propelling the animal only about one body length. The pacemaker neurons themselves are interneurons confined to the nerve rings and the excitation pathway was originally assumed [21] to be either the epithelium itself conducting in a myoid fashion, or a diffuse motor nerve net connecting the pacemaker neurons with the muscles, as had been suggested for certain Leptomedusae. Attempts to demonstrate such a net histologically in *Aglantha,* however, were unsuccessful and the possibility of non-nervous conduction in the myoepithelium could also be ruled out [18]. We already knew that there were eight motor giant axons running up from the margin into the muscle sheet (fig. 1B) and that they conducted rapidly-propagating ($<3.0\ m \cdot s^{-1}$) sodium spikes during escape swimming [15, 16]. The answer to how slow swimming is spread came unexpectedly when it was found that the same motor giant axons can also generate slowly-propagating ($<0.4\ m \cdot s^{-1}$) calcium-based spikes [25]. Figure 3B1 shows one of these calcium spikes recorded from two sites along the axon. The spike retains its low amplitude and propagates without decrement. The sodium and calcium spikes are compared in figure 3B2. The ability of the motor giants to conduct two sorts of propagated impulses is a phenomenon still without any known parallel in other organisms.

Transmission from the pacemaker neurons to the motor giants involves a slowly rising and slowly decaying EPSP (fig. 3A3). The example shown in figure 3A2 has a calcium spike developing at its apex. The threshold for production of Ca^{2+} spikes is ca. –51 mV, compared with –33 mV for Na^{+} spikes [26]. As the peak of the calcium spike lies below the threshold for sodium spikes, calcium spikes do not trigger sodium spikes; thus, the two events can function independently in the two sorts of behavior. The ionic basis of these events is further discussed below (page 13).

The muscle contractions seen in slow swimming are weaker than those seen in escape, partly due to the lower amplitude and slower rise time of the post-synaptic depolarization and partly to the restriction of excitation to the region immediately adjacent to the motor giants, as excitation does not appear to be conducted out laterally across the muscle fields in this type of swimming [18].

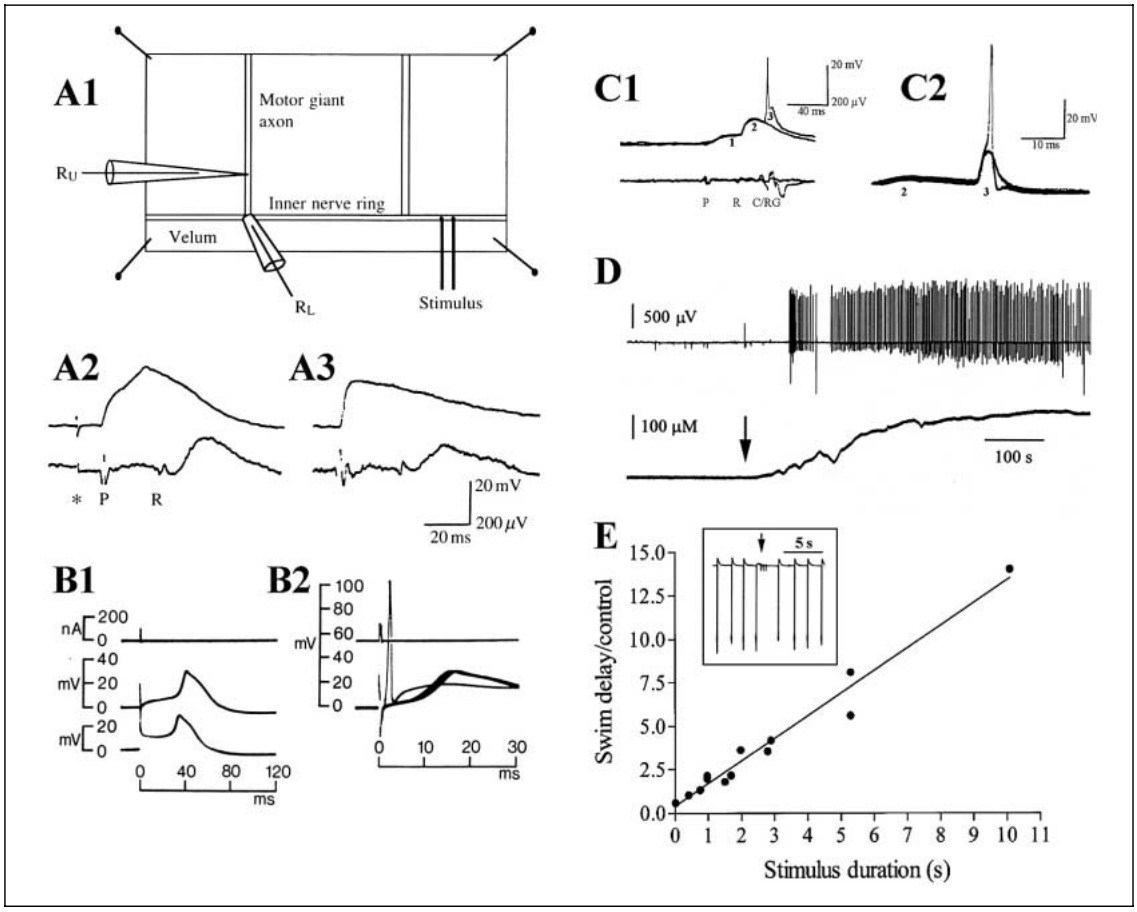

Fig. 3. Slow swimming records. **A** A typical preparation for recording from a motor giant axon is shown in A1. An intracellular electrode (R_U) is inserted in the axon. An extracellular electrode (R_L) records events in the nerve rings. Stimulation (*) of the nerve ring excited the pacemaker system causing a slow EPSP in the motor giant that generated a calcium spike (upper trace in A2). A subthreshold EPSP is seen in A3. Pacemaker events invariably trigger activity in the relay system, whose extracellular correlates are seen ca. 27 ms after the pacemaker event in both A2 and A3 (lower traces). The following slow potential represents depolarization of epithelial cells in the vicinity of the nerve rings [57]. **B** With two electrodes inserted 2 mm apart in a motor giant, a brief injection of depolarizing current through one electrode evoked a propagated calcium spike (B1). A small increase in the intensity of injected current (top trace) resulted in a sodium spike (B2). Three superimposed calcium spikes are included in this figure for comparison [25]. **C** In C1, two superimposed sweeps are shown, both following single shocks to the nerve ring recorded extracellularly (lower trace) and intracellularly from the ring giant axon (upper trace). The pacemaker, relay and carrier systems fired in sequence producing summing EPSPs (1, 2 and 3, respectively) in the ring giant that caused the latter to spike in one case. Steps 2 and 3 in a similar cascade are shown expanded in C2 [57]. **D** Induction of swimming by nitric oxide. The NO donor DEA/NO was added to the water bath (arrow) and the NO level was monitored by a NO-sensitive electrode placed beside the specimen (lower trace). When the concentration reached ca. 20 μ*M,* the animal responded by a long burst of slow swimming recorded as an electromyogram (upper trace) [70]. **E** Inhibition of swimming by epithelial impulses. The inset shows part of a regular swimming sequence (recorded electromyographically) interrupted briefly by the arrival of endodermal epithelial events evoked by stimulation (arrow) and propagated down the radial canals. The graph plots the relationship between the number of endodermal epithelial impulses (stimulus duration) and the resulting increase in the interval before the following swim, from a series of such experiments [39].

Extrinsic Factors Affecting the Output of the Slow Swimming Pacemakers

The swimming rhythm does not appear to be affected by variations in light intensity or water conditions, but these aspects have been little investigated. The absence of ocelli may not in itself mean that the animal is insensitive to light, as extraocular photosensitivity has been reported in many cnidarians [27, 28].

Swimming is strongly activated by low levels of nitric oxide (30–50 n*M*) in the water (fig. 3D). Neurons con-

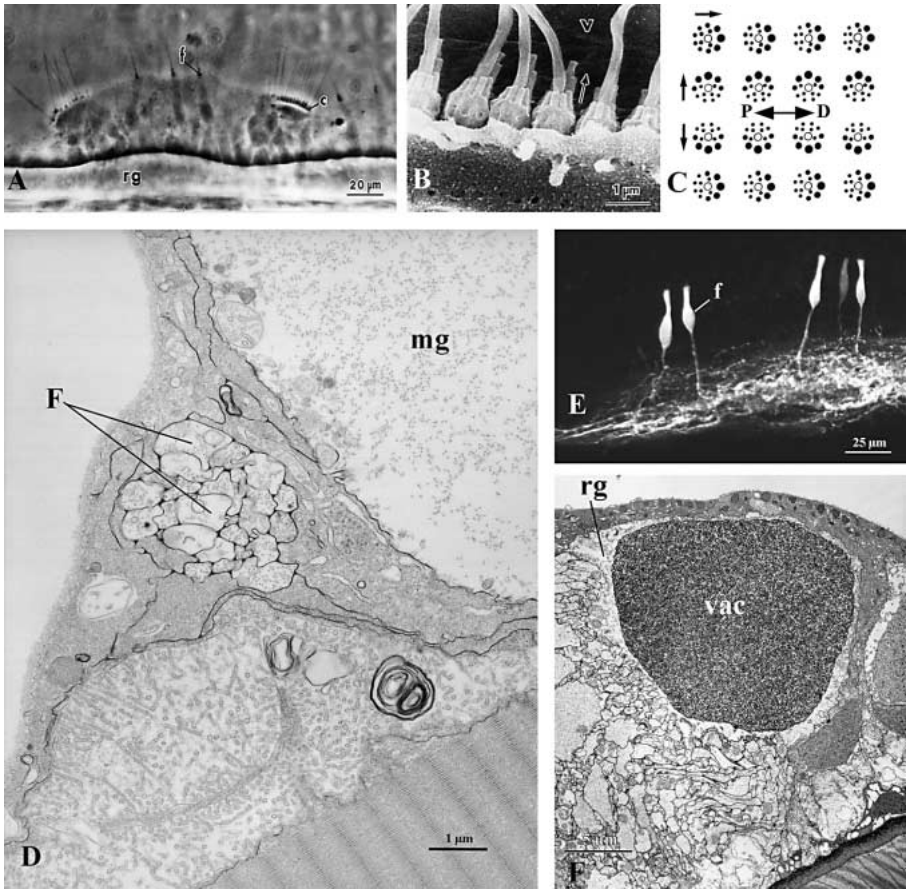

Fig. 4. Fine structure. **A** Phase-contrast image of the outer surface of the velum showing tactile combs (c), FMRFamide immunoreactive sensory cells (f) and the ring giant axon (rg) [44]. **B** Scanning electron micrograph of part of a tactile comb, showing hair cells with microvilli surrounding the cilium. The arrowhead shows the polarization of the microvillar collar toward the velum (v) [44]. **C** Orientation of hair cells on a tentacle base. Polarities are indicated by arrows and the proximo-distal axis of the tentacle is also shown (P-D) [44]. **D** Transmission electron micrograph showing a cross section through part of a motor giant axon (mg) and the bundle of small, FMRFamide immunoreactive axons (F) that mediate the pointing response of the manubrium [57]. **E** Axons in the outer nerve ring labelled with anti-FMRFamide, with sensory cells (f) [39]. **F** Transmission electron micrograph of a cross section through part of the outer nerve ring containing the ring giant axon (rg) with its electron-dense central vacuole (vac). Small axons cluster around it [57].

taining nitric oxide synthase are present in the tentacles and outer nerve ring, running in parallel with the pacemaker neurons, and it is likely that this system (NO in fig. 2) functions to modulate output of the pacemakers in nature, but precisely how is unknown [29, 70].

Like many other hydromedusae and siphonophores [for reviews, see 30–33], *Aglantha* has an excitable exumbrellar epithelium that propagates all-or-none impulses that spread across the epithelium. In the case of *Aglantha* this system (E_x in fig. 2) is not involved in the usual protective 'crumpling' response (involution of the margin). Indeed, adult *Aglantha* lack the radial and circular smooth muscles that bring this about. As in *Stomotoca* [34, 35] and *Polyorchis* [23], however, exumbrellar epithelial impulses do inhibit swimming [36]. A swimming animal making contact with another object would therefore stop swimming briefly. It is interesting that juvenile *Aglantha* have radial smooth muscles in the subumbrella, and may therefore be able to crumple. If so, they lose the ability later when their fast escape swimming responses become operational. The mechanism whereby exumbrellar epithelial impulses inhibit the swim pacemakers is not known, but in *Polyorchis*, large, long-lasting IPSPs have been recorded from the neurons during inhibition [23]. IPSPs have also been recorded from swim motor neurons in *Aequorea* [37] where they are associated with contractions of the radial muscles that overlie the swim muscle layer, but this response may be mediated by nerves (which are known to be associated with the radial muscles in several Leptomedusae [38]) rather than by excitable epithelia.

Swimming inhibition also occurs in the context of feeding (fig. 3E) [39], but here the pathway (E_n in fig. 2) is the epithelium forming the walls of the endodermal canals as discussed below, p. 16.

Directionality of Slow Swimming

Animals fishing for food generally sink passively with the bell inverted ('sink-fishing') [40]. Then, starting to swim, they veer around and swim upward ('righting'). Turning to swim upward is evidently dependent on input from the eight statocysts arranged around the margin

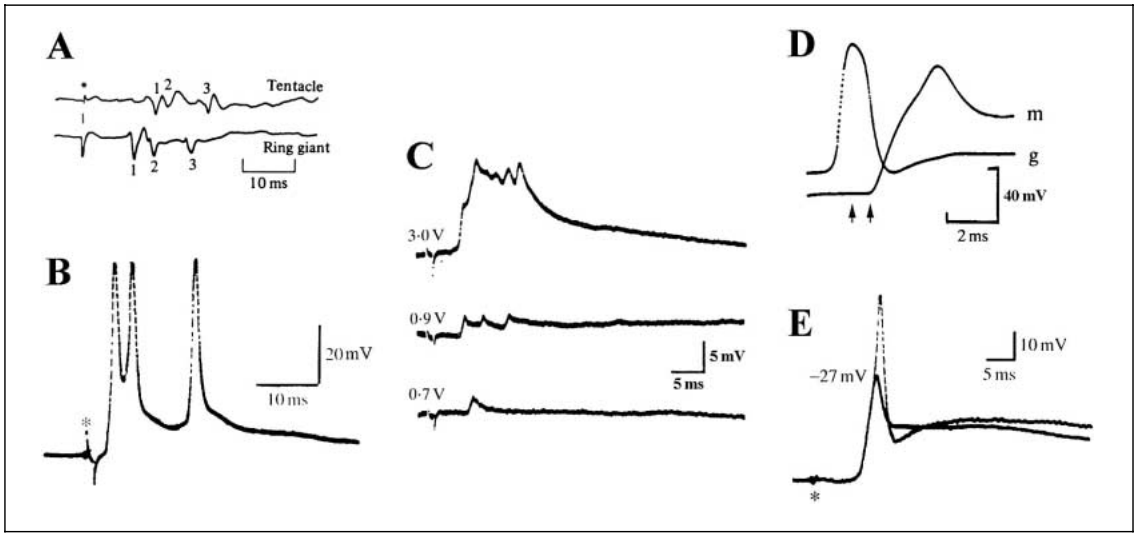

Fig. 5. Escape swimming records. **A** Extracellular recordings from a tentacle giant axon and the ring giant following a shock to the margin (*) showing similar patterns of impulses in the two [16]. **B** Burst of three spikes recorded intracellularly from the ring giant axon following a shock to the outer nerve ring [58]. **C** EPSPs recorded from the ring giant following mechanical stimuli delivered to the velum by a probe mounted on a speaker coil. Voltages applied to the coil are shown on the left [44]. **D** Simultaneous intracellular records from a motor giant axon (g) and a nearby (80 μm) myoepithelial cell (m) following stimulation of the motor giant. Arrows show the synaptic delay between the peak of the sodium spike and the start of the muscle spike [18]. **E** A shock on a motor giant generated a spike that propagated into rootlet interneurons producing either a spike or a subthreshold event in an adjacent motor giant. The subthreshold event (functionally an EPSP) is regarded as a rootlet spike attenuated by passage through gap junctions [7].

(fig. 1A) as after removal of these structures righting ability is lost [21]. The statocysts show structural features typical of gravity receptors [41] and their axons run into the outer nerve ring. There is no reason to suppose that statocyst input affects the swim pacemakers. Righting is probably brought about by asymmetric contraction of the velum during swimming as described for *Polyorchis* [42]. In both *Aglantha* and *Polyorchis*, the outer velar myoepithelium has radial muscle fibers, and is innervated by neurites from the outer nerve ring [38]. Impulses generated in activated statocysts probably excite these muscles selectively, deforming the velum on one side, but the precise way this happens has never been determined, and the system is omitted from the circuit diagram (fig. 2).

Escape Swimming

Escape swimming occurs in response to mechanical or electrical stimulation of the tentacles and of sites on the margin and subumbrella. The response has been repeatedly observed in large aquaria following contact with a predator [15] and clearly serves a defensive function. A single escape swim can propel the animal a distance equivalent to five body lengths with peak velocities up to 0.4 $m \cdot s^{-1}$ (fig. 1D). The response is mediated by rapidly-conducting giant axons. A single giant axon runs the length of each tentacle ('tentacle giant'), another runs circularly around the margin (fig. 4A, 'ring giant'), and as already noted a further set of eight 'motor giants' run radially up the subumbrella to the apex of the subumbrellar cavity (fig. 1B, C). The response evoked by stimulation on the 'outside' (outer margin and tentacles) differs from that seen following stimulation on the 'inside' (subumbrella) and the two will be considered separately.

Response to Outside Stimulation. Abrupt stimulation of one or more tentacles can evoke the escape response. Extracellular recordings [16] suggested that the tentacle giant axons were electrically coupled to the ring giant as they typically fired one-to-one with it (fig. 5A). However, the two systems do not appear to be in contact histologically [43], so the connections must be indirect, and may involve units of the carrier system (see below).

The ring giant also receives synaptic input from sensory cells located around the margin. Patterns of excitatory post-synaptic potentials (EPSPs) are recorded from the ring giant following mechanical displacements of the velum and tentacles (fig. 5C). The sensory cells responsible occur in clusters (fig. 4A, 'tactile combs') distributed

around the velum and on the tentacle bases. They resemble vertebrate hair cells in having a cilium surrounded by a collar of microvilli graded in length from one side to the other (fig. 4B). They are arranged in rows where all the cells show the same polarity (fig. 4C). Unlike vertebrate hair cells they are primary sensory neurons and send an axon into the outer nerve ring to synapse with the ring giant. Laser ablation experiments have shown that responses to water-borne vibrations depend on the tactile combs, and they are therefore viewed as hydrodynamic receptor organs [44].

The ring giant axon characteristically fires in bursts of three or more spikes (fig. 5B), but only the first of these is transmitted to the motor giants during escape swimming. It is not clear why the following spikes are not transmitted. Transmission is chemical [16, 17] with large, fast-rising EPSPs recorded in the motor giants near their bases [26], and occurs with a delay of ca. 1.6 ms. Passage between the ring and motor giants is probably not direct, but may occur through a disynaptic link. The carrier system is probably involved here as its close association with the ring giant is known from other experiments (see below).

Because the ring giant conducts rapidly (<2.6 m$\cdot$s^{-1}) and in both directions around the margin, excitation reaches all eight motor giants almost simultaneously and contraction is virtually synchronous all around the margin. It is noteworthy that *Polyorchis*, a much larger animal, achieves synchrony in a completely different way, involving progressive reduction of synaptic delay as the impulse travels round the margin [45]. The motor giants generate sodium spikes (fig. 3B2) that propagate at velocities up to 4 m$\cdot$s^{-1}. They synapse directly with cells of the swim myoepithelium in their immediate vicinity through structurally polarized, chemical-type junctions [14]. Transmission is calcium-dependent and junctional delay has been measured at 0.7 ± 0.1 ms, making them among the fastest known invertebrate synapses [18] (fig. 5D). The motor giants are dye- (and presumably electrically-) coupled to a set of lateral neurons (fig. 1B) that run out sideways and excite interradial areas of the myoepithelium through chemical junctions [18]. The chemical identity of transmitters in the swim pathways has not yet been determined.

The combination of rapid conduction in giant axons, short delays at the neuromuscular junctions and rapid development of tension in the myoepithelium ensures a short overall response time and fast escape from potentially damaging sources of stimulation. Response latency, measured from stimulus to first detectable movement, is about 10 ms [46], which compares favorably with fast-start response latencies of many fishes [47].

Response to 'Inside' Stimulation. Stimuli applied to the subumbrella may result in excitation of one or more of the motor giant axons. There are no mechanoreceptive sensory cells associated with these axons, but they lie very close to the surface and they and/or the lateral neurons coupled to them are probably stimulated by direct contact, as might occur when foreign bodies are sucked into the subumbrellar cavity. Stimulation of a motor giant anywhere along its length can evoke a sodium spike that propagates to all the other motor giants, evoking synchronized escape swimming. The pathway around the margin in this case is not the ring giant but a system of rootlet interneurons that run in the inner nerve ring (fig. 1B). Each motor giant is electrically coupled to rootlet interneurons that run out laterally on either side within the inner nerve ring, where they mingle in a zone of overlap with rootlet interneurons 'belonging' to the neighboring motor giants. Transmission between rootlet interneurons is chemical [7] and results in generation of action potentials that propagate to the next motor giant, and so on around the ring. Rootlet interneuron input can be recorded in motor giants close to the junctional region as attenuated action potentials (fig. 5E) – attenuated after passage through the gap junctions that occur here [48]. Though purely electrical in origin, these attenuated spikes function like the fast-rising, chemical EPSPs produced by ring giant input, generating sodium spikes [17] and they bear a striking (if superficial) resemblance to the chemical EPSPs in waveform.

Conduction along the rootlet interneuron chain occurs at 0.5 m$\cdot$s^{-1}, more slowly than along the ring giant, so response latency in this form of the escape response is probably longer than with outside stimulation, although this has not been measured.

As shown in the circuit diagram there are two-way excitatory interactions between the pacemaker and rootlet interneuron systems. Pacemaker impulses generate slow EPSPs in the rootlet interneurons but spikes in the rootlets generate spikes in the pacemaker neurons on a one-for-one basis. Thus, when the rootlet interneuron pathway is excited during escape swimming, the pacemaker system is also excited. The pacemaker neurons may fire repetitively if stimulated close to the time when they would normally generate a spontaneous burst. This explains why escape swims, whether due to outside or to inside stimulation, are sometimes followed by bursts of slow swimming.

Ionic Basis of Na^+ and Ca^{2+} Spikes in the Motor Giants

The 'slow' EPSPs recorded from motor giants that represent pacemaker input depolarize the axon from –70 to –51 mV, the threshold for initiation of calcium spikes. This corresponds to the voltage at which calcium current starts to flow in voltage clamp experiments [49] and in axon membrane patches [26]. In contrast, the 'fast' EPSPs seen during escape responses depolarize the axon to –32 mV, corresponding to the voltage at which inward sodium current starts to flow [26]. As noted earlier, the peak of the calcium spike lies below the threshold for sodium spikes, so calcium spikes do not set off sodium spikes. All evidence to date from drug experiments and electrophysiology point to T-type Ca^{2+} channels as the portals for calcium influx in calcium spike electrogenesis. Another type of Ca^{2+} channel may play a role at neuromuscular synapses.

Repolarization involves a family of A-type potassium channels, of which three categories have been distinguished [49, 50]. They have similar conductances, suggesting that they evolved by gene duplication, but they differ in their voltage dependencies and inactivation kinetics, and are accordingly referred to as 'fast', 'slow' and 'intermediate'. Rapid activation of the fast K^+ channels evidently serves to cut short the inward Ca^{2+} current near the peak of the calcium spike preventing the latter from reaching the threshold for sodium spikes. The same channels, together with the more slowly activating species, contribute to the repolarization of the sodium spike.

Coordinated Tentacle Contractions Accompanying Swimming

Typical specimens of *Aglantha* have 60–80 tentacles arranged around the margin. When the animal is fishing for food it stops swimming and sinks with the tentacles extended on all sides. Movement relative to the water mass is assisted by beating of the powerful cilia arranged in rows on either side of the tentacles. The cilia are borne on epithelial cells equipped with basal muscle processes that form part of the general, longitudinal muscle layer, and when these muscles contract, the cilia simultaneously undergo arrest [51]. Tentacles can respond to local stimuli by flexing independently, but during swimming they all contract. *Aglantha* differs markedly from other hydromedusae in the way these contractions are coordinated. Most medusae have a single, marginal conduction system that extends into the tentacles and is dedicated to coordination of the tentacles. Its electrical correlates, originally termed 'marginal pulses' [52], were later described under several other names [2]. In *Polyorchis* it is termed the 'B' system [53– 55]. Nothing comparable to this system exists in *Aglantha*. Further, the tentacle contractions seen during escape behaviour differ markedly from those seen during slow swimming and are mediated by different neural pathways. Finally, *Aglantha* differs from all other known hydromedusae in having striated muscles in the tentacles [16]. These probably evolved in response to a need for rapid contractility during escape behavior, but they are responsible for the graded contractions seen at other times, being the only muscle fibers present.

The coordinated tentacle contractions observed during swimming are probably significant as a way of reducing drag and making locomotion more efficient, but the tentacles have an 'autotomy joint' at their bases and readily detach when tugged sharply [43], so the contractions that occur during swimming, particularly during escape swimming, may serve a secondary role in reducing the risk of 'accidental' autotomy.

The tentacles have two physiologically distinct conduction systems. Small potentials are associated with slow conduction at $<0.2\ m \cdot s^{-1}$ in a network of smaller neurites and mediate the slow contractions of graded amplitude seen in non-escape contexts. Larger events are conducted at $<0.9\ m \cdot s^{-1}$ in the tentacle giant axon and trigger rapid, all-or-none 'twitch' responses. The two systems are here termed the slowly-conducting tentacle system and the tentacle giant system, respectively [57, 58]. We will now consider how these systems are brought into play during the two sorts of swimming.

Tentacle Contractions Accompanying Slow Swimming

The impulses generated by the pacemaker neurons during slow swimming trigger events not only in the motor giants but also in interneurons of the relay system (fig. 3A2, A3) that runs in parallel with it and this will, in the simplest scenario, be followed by excitation of the slow tentacle system resulting in orally-directed tentacle flexions. The amplitude of the tentacular response increases with each swim in a series of slow swims, eventually resulting in all the tentacles being tightly curled in close to the margin. This appears to be the basic role for the relay system – to bring about tentacle contractions during slow swimming.

Surprisingly, twitch contractions of the tentacles are sometimes seen during slow swimming, along with the graded, or tonic, sort. These are identical to the synchronized contractions seen during escape swimming and like

them are conducted round the margin by impulses in the ring giant axon and down the tentacles in the tentacle giant system. It appears that the hard and fast distinction between escape and non-escape circuitry breaks down in this case as we normally associate ring giant activation exclusively with escape responses. The mechanism is described below.

Tentacle Contractions Accompanying Escape Swimming following Outside Stimulation

As already noted, tactile and vibrational stimuli applied to the tentacles or margin lead to excitation of the ring giant axon. Events conducted around the margin by this unit automatically excite the tentacle giant system in all the tentacles, resulting in a powerful, unified, twitch response that slightly precedes the onset of swimming.

Tentacle Contractions Accompanying Escape Swimming following Inside Stimulation

We have already seen that impulses generated in the rootlet interneuron system during escape swimming trigger activity in the pacemaker system, which may respond repetitively, producing a series of slow swims following the escape swim. This is not the end of the story, as impulses in the pacemaker system are always followed one-for-one, after a short delay, by impulses in the relay system (fig. 3A2, A3). Intracellular recordings show that the ring giant receives input in the form of EPSPs from both these sources and also from a third source, the carrier system which fires following the relay system, again after a short delay (fig. 3C1, C2). The EPSPs from these three sources appear sequentially in the ring giant, and the depolarizations sum, sometimes to spike threshold at about –46 mV. A cascade of summing inputs from three sources, firing in sequence, is evidently necessary for spike production in the ring giant, no doubt because of the axon's large size and high membrane capacitance [7, 5, 58].

We do not understand why the ring giant sometimes spikes and sometimes fails to spike in these circumstances, but whether it does so or not determines whether the resulting tentacle response will be of the fast-twitch or slow-graded sort. If the ring giant spikes, the tentacles will show the concerted twitch response. If it does not, and if the pacemaker system continues to fire repetitively, the tentacles will still show graded slow contractions owing to the direct activation of the slow tentacle system by the relay system. Sometimes the response seen appears inappropriate to the behavior. While a twitch contraction of the tentacles during slow swimming would be harmless, a single, slow contraction during escape swimming would seem virtually useless as it would do little to reduce tentacular drag. It might however reduce the risk of autotomy as the muscles in the autotomy zone would have developed some tonus.

Of the interneurons involved in communication with the ring giant, the carrier system has proved hardest to characterize. It can conduct slowly on its own but conducts rapidly at the same velocity as the ring giant when the latter conducts impulses. When firing in synchrony with the ring giant, its electrical correlates tend to be submerged in those of the latter so it is indistinguishable in extracellular recordings, or forms a minor part of a combined carrier/ring giant event (e.g. fig. 3C1). EPSPs representing carrier input are seen in intracellular recordings and are diminished by treatment with divalent cations, indicating chemical transmission. The system gets its name from the fact that it can 'carry' impulses around regions where the ring giant has been damaged. The impulses reappear in the ring giant on the other side. It seems to provide an input link between the ring giant and other systems, as in the cascade referred to above, and on the output side by transmitting excitation from the ring giant to the motor giants during escape swimming after outside stimulation, where the latency seems to call for a disynaptic connection. The carrier system also probably provides an input-output link between the ring giant and the tentacle giant system.

Piggyback Interactions

Piggybacking is a process seen in several hydrozoans where events propagated in one conduction system travel at an accelerated rate when a second, faster system running in parallel with it, is also excited. As in the children's game, one system rides 'piggyback' on the back of the other. The process was first noted in the stem of a siphonophore where the endoderm was found to be an excitable epithelium that conducted slowly on its own at 0.3 $m \cdot s^{-1}$. When giant neurons running in the ectoderm were simultaneously excited, propagating at velocities up to 3.0 $m \cdot s^{-1}$, conduction in the endoderm was accelerated to almost the same value. The ectoderm and endoderm were found to be connected by transmesogloeal bridges, with gap junctions between the epithelial cells of the two layers. The explanation advanced to explain piggybacking in this case was that events conducted in the giant axons depolarized the ectodermal myoepithelium through conventional synapses and that the depolarizations spread through gap junctions to the endoderm, assisting the forward spread of action currents, and hence increasing the speed of impulse propagation in the latter [59].

In jellyfish nerve rings, we have a similar situation with several conduction systems running in parallel. It has long been recognized that the individual axons lack glial sheaths [1, 60], although groups of them may be loosely bundled within epithelial processes. In *Aglantha,* piggyback interactions have been observed between several systems, as shown in figure 2 by squiggly-shafted arrows (an iconographic reference to a pig's tail). To take one example, the relay system conducting on its own never showed a conduction velocity exceeding 0.1 $m \cdot s^{-1}$, but when the pacemaker system was simultaneously active, relay velocities increased to 0.24 $m \cdot s^{-1}$, and when both the pacemaker and ring giant systems were active, relay velocity increased to 0.41 $m \cdot s^{-1}$ [57]. Likewise, the carrier system conducting on its own showed a conduction velocity of <0.5 $m \cdot s^{-1}$ in a preparation where it conducted at <2.0 $m \cdot s^{-1}$ in the piggyback mode, carried on the back of the ring giant [58].

The mechanisms for piggybacking in *Aglantha* are unclear, but observations on the effects of various drugs [57] suggest that it not always mediated either by gap junctions or by chemical synapses but may involve some sort of external 'field effect' [61, 62]. While such interactions between functionally distinct neuronal subsets might be explained away as an insulation defect related to the lack of proper glial sheaths, it appears that at least in some cases the process has been put to good use. Piggybacking may be important in the case of ring giant activation in escape behavior set off by inside stimulation where the ring giant requires sequential input from the pacemaker, relay and carrier systems within a restricted time frame in order to reach spike threshold. Piggybacking may help maintain these inputs in an optimal time relationship.

Pathways Mediating Feeding Behavior

When fishing for food, *Aglantha* typically sinks inverted with outstretched tentacles. Prey contacting a tentacle are captured by discharge of nematocysts and held to the tentacle by the discharged thread. The tentacle then bends toward the margin ('oral tentacle flexion'). On reaching the margin, the food is held there until transferred to the mouth, which lies at the tip of the muscular, prehensile manubrium (fig. 1A). The manubrium bends across toward the point where the food is located (the 'pointing' response described in other medusae [35, 63], while the oral lips expand ('lip flaring') and apply themselves to the prey. The prey is then engulfed and digested. Animals which were swimming at the start of feeding stop doing so while food is being transferred to the manubrium and engulfed ('swimming inhibition'). The action systems involved in these four steps [39] will now be considered.

Oral Tentacle Flexions. These movements are of the slow type, mediated by the slow tentacle system described above. The flexions affect only those tentacles directly stimulated, others remaining extended. Neurosensory cells bearing short sensory processes occur in the slow tentacle net and probably trigger the flexions.

Pointing. Bundles of small axons run radially from the margin to the manubrium where they selectively innervate muscle bands located in the walls of the manubrium. There are eight such pathways, each with its 'own' muscle band. These pathways in *Aglantha* have been termed the flexion system (F, in fig. 2), because they mediate unilateral manubrial flexions in the pointing response. The flexion system originates at least in part from sensory cells (fig. 4A, E), referred to as type 2 sensory cells [38, 41] or F cells [here and 39, 44], which are located at the margin. Sensory neurons in the tentacles form a separate nerve plexus there (TF in fig. 2) that appears to be part of the same system. Though originating from sensory cells, flexion axons function as a motor pathway in exciting the pointing muscles. The radial flexion tracts are seen as bundles of small axons in TEM sections (fig. 4D). They and the flexion cells show FMRFamide-like immunoreactivity, another reason for the F designation. They are interconnected at the margin by axons running circularly in the nerve rings. Impulses through-conduct via these circular connections to all eight tracts, but the most strongly excited manubrial muscles are those closest to the site of the stimulus, and thus pointing in that direction results.

The presence of FMRFamide-like immunoreactivity in the flexion system neurons, as detected by fluorescent and immunogold labelling [38, 64], suggests that peptidergic transmission occurs at the neuromuscular junctions involved in pointing. The peptide in question has not been sequenced, but RF- and related short-chain peptides are widely distributed through the Cnidaria and have frequently been implicated as neurotransmitters or neuromodulators [65].

Lip Flaring. At the same time as the manubrium points to a site of prey capture, its lips flare wide open, preparatory to attaching to the prey. Lip flaring is a symmetrical response involving the ectodermal longitudinal muscles on all sides of the manubium. The response is mediated not by nerves but by the epithelium forming the walls of the endodermal radial canals, termed the E_n pathway (fig. 2). It is not known how impulses cross between the

endoderm and the ectoderm, but epithelial bridges cross the intervening mesogloeal layer, and nerves run in both layers of the manubrium. Conduction occurs circularly both at the margin (in the ring canal) and in the manubrium itself so endodermal epithelial impulses initiated at any point spread throughout the entire system. The system conducts centripetally in response to food at the margin, but later during feeding it conducts centrifugally as bursts of endodermal epithelial impulses are generated locally in the manubrium during ingestion. The lip flarings seen at this stage help spread the lips around the food. These bursts are probably generated by neuronal pacemakers even though they then propagate in an epithelium. Two-way neuro-epithelial interactions are known for a number of other animals [32].

Swimming Inhibition. In attempts to maintain captive *Aglantha* by feeding them with brine shrimp larvae it was noticed that animals which were swimming stopped while ingesting food. Experiments later showed that swimming could be arrested by artificially stimulating the endodermal epithelial conduction system (fig. 3E). The duration of inhibition depends on the number and frequency of endodermal epithelial impulses arriving at the margin, and some degree of inhibition persists even after swimming has been resumed. As noted, endodermal epithelial impulses can propagate in either direction along the radial canals and are generated in bursts during ingestion. While it is not clear precisely how the impulses inhibit the swim pacemakers, it is known that there are trans-mesogloeal processes connecting the endo- and ectoderm at the margin in the vicinity of the nerve rings, and that some groups of nerves in the nerve rings are enveloped by processes of epithelial cells. Neither gap junctions nor synapse-like structures have been seen where the epithelial cells contact the nerves however.

We have seen earlier that epithelial impulses generated in the exumbrellar ectoderm inhibit swimming in *Aglantha* [36] and that intracellular recordings from the pacemaker neurons in *Polyorchis* showed hyperpolarizations during swimming inhibition [23]. It seems likely that whatever mechanism mediates the inhibition in these cases also mediates it in the case of swimming inhibition during feeding. However, endodermal epithelial impulses propagated in the canals during feeding do not spread across to the ectoderm at the margin or travel up the exumbrella, nor vice versa, so the two epithelial pathways are shown separately in figure 2.

Conclusions

Evolution of Escape Circuitry

Many of the features peculiar to *Aglantha* can be seen as adaptations to life in the competitive mid-water environment. Observations from a manned submersible in the waters around Vancouver Island leave a vivid impression of the dense populations of euphausiids, copepods and other crustaceans living at the very same depths where *Aglantha* are most concentrated [10]. *Aglantha*'s acute vibrational sensitivity and unique escape behaviour may well have evolved as an adaptation to life in heavily populated mid-water zones, where they would help reduce the risk of damaging contact with their numerous, spiny crustacean cohabitants. Indeed, we have observed escape responses occurring in the natural habitat following chance contact with crustaceans [author, unpubl.]. On the other hand, *Aglantha* lacks ocelli, shows no 'shadow response' and lacks visual neural circuitry of the sort described for *Polyorchis* [55, 66–68], a species that lives much closer to the surface.

Aglantha has evolved a special set of components enabling it to swim and to contract its tentacles in two fundamentally different ways (escape and non-escape) and this has required wholesale modification of the basic systems which we assume were inherited from the common ancestor. Of the interneuron systems described here, the pacemaker system can be seen as a basic medusan component inherited more or less intact, but all the other neural sub-systems are unique to *Aglantha*, or have been modified to the extent that their origins can no longer be recognized. Even the pacemaker system has undergone drastic modification of its input-output relationships. Its primary output is no longer to the swim muscles (which have lost the ability for myoid conduction) but rather to neural components in the slow swimming motor pathway. At the same time it is postsynaptic to the rootlet interneurons, and so is excited during escape swimming as well as during slow swimming. In its turn, it provides input to the relay system, causing it to spike, and to the carrier/ring giant system, causing EPSPs which may sum with those due to relay input in bringing the ring giant to spike threshold.

The relay system can be seen as a key component required for activation of the slow tentacle system and thus for bringing about graded tentacle contractions during slow swimming, but it has the additional property of exciting the carrier/ring giant system. If the latter responds by spiking, the tentacle giant system will be activated, resulting in twitch contractions of the tentacles. We

see the carrier system as an adjunct to the ring giant mediating the latter's interactions with other systems, and with no known counterpart in other medusae.

Turning to the tentacles, the tentacle giant system has no counterpart in other medusae but presumably evolved, along with the striated muscles, to provide for short-latency, twitch contractions during escape behaviour. The slow tentacle system may represent a relic of the system that provides for coordinated tentacle contractions in other medusae [references in 2], but if so it has lost those portions running in the outer nerve ring that elsewhere interconnect the tentacles and is reduced to the status of a local action system.

The ring, motor and tentacle giant axons are all unique to *Aglantha*. The ring giant is highly peculiar in being a torus, the interior occupied by a fluid-filled vacuole [16]. The possibility has been suggested that it evolved from an excitable epithelium rather than from nerves [69], but this is quite uncertain. The tentacle giants also have a large central vacuole running their entire length [51]. The motor giants are unique in their ability to conduct two sorts of action potential, the only nerves known to be capable of this feat.

Despite their enigmatic origins and unusual, specialized features, these axons endow *Aglantha* with a startle response that bears comparison with Mauthner-mediated C-start responses of fishes. In one respect, however, unless perhaps our findings made under lab conditions are not truly representative of normal behaviour, the control of tentacle contractions during swimming seems less than 100% efficient. While the concerted twitch responses seen with outside stimulation of escape swimming are predictable and efficiently serve the purpose of reducing drag prior to the initiation of the violent swimming contraction, similar contractions are sometimes seen during slow swimming when one would simply expect slow, graded contractions. Further, in the case of inside stimulation leading to escape swimming, the tentacle contractions may be of the inappropriate, slow sort rather than fast, concerted twitches. Even where the twitch response is seen, it ensues *after* the initiation of the swim rather than before it owing to the cumulative delays involved in serial activation of the pacemaker, relay and carrier/ring giant systems. Its effectiveness in reducing drag must therefore be much reduced. One can only conclude that natural selection has not yet completed the task of sorting out all the problems raised by the introduction of special escape circuitry and the need to integrate these components with the existing slow circuitry.

No such problem applies to the activation of the swim muscles during the two sorts of swimming – the distinction is always a clear-cut one. The fast response depends on sodium spikes in the motor giants and the slow one on calcium spikes and the two do not overlap. The tentacle responses in contrast rely on interactions between several different conduction systems which, by nature, involves a more 'noisy' type of decision-making process.

Aglantha's Interesting Use of Excitable Epithelia

The loss of protective, 'crumpling' behavior of the sort seen in other hydromedusae is probably related to *Aglantha*'s development of a rapid, escape swimming response. It no longer needs both means of protecting itself. At the same time, it has retained epithelial excitability in the exumbrella and uses this pathway to inhibit swimming in the event of collision with foreign objects. Finally, it has retained excitability in the endodermal radial canals (originally involved in crumpling) and uses these pathways to bring about lip flaring during feeding, and to inhibit swimming while the manubrium is ingesting food. It would seem that *Aglantha* has effectively commandeered components left over from an action system it no longer needs and redeployed them for use in a very different context.

Aglantha *as a 'Model'*

Choice of animals for neurophysiological work (e.g. *Sarsia*, *Stomotoca*, *Aequorea*, *Polyorchis*) has been influenced more by factors of convenience, such as their predictable appearance at certain sites and seasons, than by how 'typical' they are, and *Aglantha* is no exception. It is one of the few holoplanktonic species that can be counted on to appear every year close to a major marine station. Its special neural and muscular adaptations as described in this review are probably also present in other rhopalonematid medusae but not so far as we know in members of other Families [70]. This may limit *Aglantha*'s usefulness as a model, but members of all medusan families have their own peculiarites and not enough species have been examined in sufficient depth for it to be easy to pick any one species as the best model for the group as a whole.

This being said, it must be admitted that *Aglantha* is quite unlike the species named above and is indeed something of 'a special case' [6]. The presence of giant axons and the substitution of neural for myoid conduction in the swim muscles set it apart. The dual innervation of both the tentacular and swim muscles and the way in which the tentacles are coordinated are also unique features. It is not

even clear that *Aglantha* conforms to the well-known paradigm established for other hydromedusae (fig. 7 in ref. [22]) in which the various neural subsets are composed of electrically coupled units, with chemical synapses restricted to interfaces between subsets. It is customary to think of the pathways in medusan nerve rings and elsewhere as 'compressed nerve nets' [2], but if the compact bundles of axons comprising the flexion system are anything to go by, this may not apply in the case of *Aglantha*. These axons innervate the pointing muscles, traveling from the margin to the manubrium. They do not appear to synapse or make gap junctions with one other, and can be regarded as 'a nerve' in the same way that we speak of the vagus or sciatic nerve [39].

It is not just *Aglantha*'s neuromuscular organization that sets it apart, but its whole biology. All the 'conventional' species named above live in relatively shallow coastal waters, being tied to sessile hydroid stages. *Aglantha* has no hydroid stage and is often found thousands of miles from the nearest land. It has evolved for a very different sort of life. It has been extremely instructive to work on, not because it typifies hydromedusae as such, but because it shows what the cnidarian body plan is capable in terms of nervous organization. For a diploblastic, acephalic animal it has an astonishingly sophisticated nervous system. One must concur with Satterlie [6] that 'any reticence in acknowledging the complexity of the central nervous system of these animals, and of their integrative abilities, should be abandoned'. Much still remains to be explored and future workers will find the task a rewarding one.

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Review

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The Insect Frontal Ganglion and Stomatogastric Pattern Generator Networks

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Key Words
Central pattern generator · Feeding behavior · Frontal ganglion · Locust · *Manduca sexta* · Molting · Neuromodulation · Stomatogastric nervous system

Abstract
Insect neural networks have been widely and successfully employed as model systems in the study of the neural basis of behavior. The insect frontal ganglion is a principal part of the stomatogastric nervous system and is found in most insect orders. The frontal ganglion constitutes a major source of innervation to foregut muscles and plays a key role in the control of foregut movements. Following a brief description of the anatomy and development of the system in different insect groups, this review presents the current knowledge of the way neural networks in the insect frontal ganglion generate and control behavior. The frontal ganglion is instrumental in two distinct and fundamental insect behaviors: feeding and molting. Central pattern-generating circuit(s) within the frontal ganglion generates foregut rhythmic motor patterns. The frontal ganglion networks can be modulated in-vitro by several neuromodulators to generate a variety of motor outputs. Chemical modulation as well as sensory input from the gut and input from other neural centers enable the frontal ganglion to induce foregut rhythmic patterns under different physiological conditions. Frontal ganglion neurons themselves are also an important source of neurosecretion. The neurosecretory material from the frontal ganglion can control and modulate motor patterns of muscles of the alimentary canal. The current and potential future importance of the insect stomatogastric nervous system and frontal ganglion in the study of the neural mechanisms of behavior are discussed.

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Introduction

The insect nervous system has been widely and successfully employed as a model system in the study of the neural basis of behavior [e.g. 1–4]. Hoyle [1] has noted the outstanding importance of the insect model system for a comparative neurophysiological approach to neural net-

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work analysis (as in this special issue of *Neurosignals*). The considerable potential of insect models arises, on the one hand, from the great accessibility and simplicity of the insect nervous and neuroendocrine systems. Both systems have been amply studied and are well characterized in insects [4–6]. On the other hand, insects are capable of demonstrating complex and very carefully controlled behavioral processes that are relatively easily studied in the laboratory. The many similarities and common principles shared by the nervous systems of simple and higher organisms are by now a well-known and widely accepted fact [e.g. 7, 8].

A second key aspect of research on insect neural networks is that insights into the basis of insect behavior have important practical consequences. The arsenal of safe and cost-effective insecticides is shrinking for various reasons (e.g. insecticide resistance and unacceptable side effects). New strategies for insect pest management, more effective and above all ecologically safe ones, pose an ongoing challenge. Detailed knowledge of the neural and neuroendocrine mechanisms underlying insect behaviors can serve the urgent need to define alternatives targets.

The insect stomatogastric nervous system (also referred to as the enteric or stomodeal nervous system in early work) is present, in some form or another, in all known insect species [9, 10]. It serves to innervate the anterior parts of the insect digestive tract [11–21]. Ample work and very rich literature has been dedicated to this system in Crustacea ([22], see also the article by Hooper, pp. 50–69, in the present issue). Mainly due to its small cell number and (relatively) simple behavioral output, the stomatogastric nervous system in lobsters and crabs has served for several decades as a leading model in the study of the neural control of rhythmic behavior. Our knowledge of the equivalent insect system (the question of homology is an important one but beyond the scope of the current work) is lagging far behind. Furthermore, as the stomatogastric nervous system is present in all higher invertebrates, starting from annelids [23–26], it offers a very suitable and attractive case for evolutionary or comparative investigations of the insect nervous system.

This review summarizes the present knowledge on the insect stomatogastric nervous system and, specifically, one of its principle components, the frontal ganglion (FG). Following a short description of the anatomy of the system in different insect groups, I briefly present aspects of the development of the insect stomatogastric nervous system. Both issues have been covered previously in detailed reviews (see below). The current paper focuses on the neural basis for the control of two fundamental behaviors in an insect's life: feeding and molting. As will be described, the stomatogastric nervous system, and specifically the FG, plays a critical role in both behaviors. The current state of our knowledge of the neural networks that generate foregut rhythmic movements will be presented. Finally, the importance of neuromodulation and neurosecretion in the insect stomatogastric nervous system will be discussed.

The Insect Stomatogastric Nervous System: Gross Anatomy and Development

The anatomy of the stomatogastric nervous system in insects reflects its function and thus varies mainly according to the mode of feeding of the species. As would be expected, the system is generally reduced in liquid-feeding insects, where foregut movement is mainly myogenic [27, 28] but is more evident in insects feeding on solid foodstuffs, where movements of the foregut are more complex, i.e. both myogenic and neurogenic [29–32]. In the case of holometabolous insects, where the mode of feeding is closely tied to the insect's developmental stadium, we should also expect some changes in the stomatogastric nervous system along with the insect's ontogeny. According to Snodgrass [33], 'it is impossible to give a general description applicable to all its (the stomatogastric nervous system) numerous variations in different insects'.

Detailed morphological studies of the stomatogastric nervous system of specific insect groups were first provided by Orlov [11, 34] for the larvae of the beetle *Oryctes*. An anatomical description was also given by Willey [12] for *Periplaneta americana* and other Blattaria. Dando et al. [16] first described the system in *Schistocerca gregaria*, and Kirby et al. [20, 21] in *Acheta domesticus*. Investigations of the stomatogastric nervous system of lepidopterous insects *(Manduca sexta)* were provided by Borg et al. [35] and Bell et al. [36]. These and others were reviewed in detail by Penzlin [10] and Chapman [37]. More recently, the stomatogastric nervous system in some Dipteran species [38–40] and in *Apis mellifera* [41] was also described.

In all the studied insects the stomatogastric nervous system consists of a series of small ganglia that are closely associated with the brain, the corpora cardiaca and corpora allata and the anterior portion of the gut (fig. 1). The FG is a principal component of the stomatogastric system in most insect orders [10, 37], accordingly, this ganglion has attracted much research. The best detailed account of

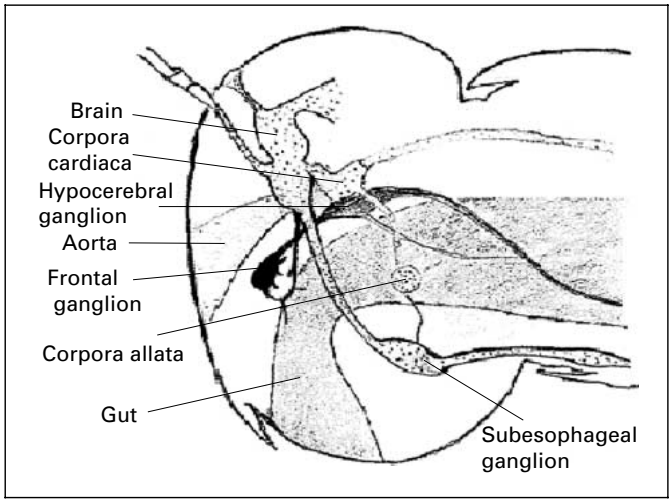

Fig. 1. Sectional diagram of an Orthopteran insect head, showing the position of the cephalic stomatogastric ganglia, the frontal and hypocerebral ganglia (black) relative to the major neural and endocrine centers. Modified from Ayali et al. [45].

the cellular structure of the insect FG was given for *P. americana* [42–44] and *Acheta* [20, 21].

Two insect preparations which, in recent years, have been at the focus of neurophysiological studies of the stomatogastric nervous system will be discussed in greater detail below. Ample work on the different Orthopteran species has set the ground for recent investigations of the FG of the desert locust *S. gregaria* [45–47], and recent studies on the tobacco hornworm *M. sexta* [48, 49] are based on previous work on lepidopterous insects.

In locusts, the FG lies in the forehead, on the dorsal side of the pharynx, in front of the brain. It is connected to the tritocerebrum of the brain by the paired frontal connectives (fig. 2a). Posteriorly, a recurrent nerve passes from the FG along the pharynx, under the brain and over the dorsal side of the esophagus. It branches onto the dilator and constrictor muscles of the pharynx, and ends in the hypocerebral ganglion which is closely associated with the corpora cardiaca. Additional three pairs of efferent nerves – the anterior (APN), median (MPN) and posterior pharyngeal nerves (PPN) – branch onto the dilator muscles of the gut in a rostrum to caudal order, making the FG the major source of foregut muscles innervation (fig. 2a). The median recurrent nerve gives rise to paired lateral esophageal nerves, which innervate the more posterior muscles of the esophagus and terminate on the crop in paired ingluvial ganglia. The FG is encased by a neural lamella. It is characterized by a central neuropil surrounded dorsally and laterally by a single or double layer of neurons [see figure 1 in 45]. The cell bodies are 25–50 μm in diameter. Their number is estimated to be about 100, depending on the exact methods used [18, 19].

The FG in both larval and adult *Manduca* is connected to the tritocerebrum by the paired frontal connectives (fig. 2b). Two additional nerves exit the *Manduca* ganglion; a single recurrent nerve that runs posteriorly from the FG to innervate the muscles of the pharynx and esophagus, and an anteriorly directed frontal nerve which innervates the buccal musculature. Unlike the locust, the *Manduca* stomatogastric nervous system does not feature a hypocerebral ganglion after the embryonic stage [48]. During adult development there is much rearrangement of foregut musculature accompanying the formation of the cibarium. In adult moths, the FG innervates the muscles of the cibarial pump. The frontal nerve innervates most of the pump dilators and the recurrent nerve innervates the pump compressors (fig. 2b); [49]. In *Manduca* fifth-instar larvae and adults, the FG is 160 μm in diameter and contains about 35 neurons, arranged in a single layer. The neurons range in diameter from about 20 to 45 μm, and a number of them have been identified in both larvae and adults [48–50].

A growing number of studies present the insect stomatogastric nervous system as a model for nervous system development. Most research on insect stomatogastric system development employed either *M. sexta* [51–55], or *Drosophila melanogaster* [56–58]. Ganfornina et al. [59] focused on the embryonic development of the locust stomatogastric nervous system. Using a number of specific molecular markers, these authors studied morphogenesis and some aspects of neuronal differentiation in the locust system. In his thorough review, Hartenstein [60] reports that the insect stomatogastric nervous system is derived from a small neuroectodermal placode located in the foregut. All cells within this placode give rise to neural cells whose precursors migrate and finally re-aggregate to form the different stomatogastric system ganglia.

Physiological Role of the Insect Stomatogastric Nervous System and Frontal Ganglion

In his review article, dedicated primarily to the structure of the stomatogastric nervous system, Penzlin [10] writes: 'Much more experimental work is necessary to gain a better consolidated insight into the control function of the stomatogastric nervous system'. More than a de-

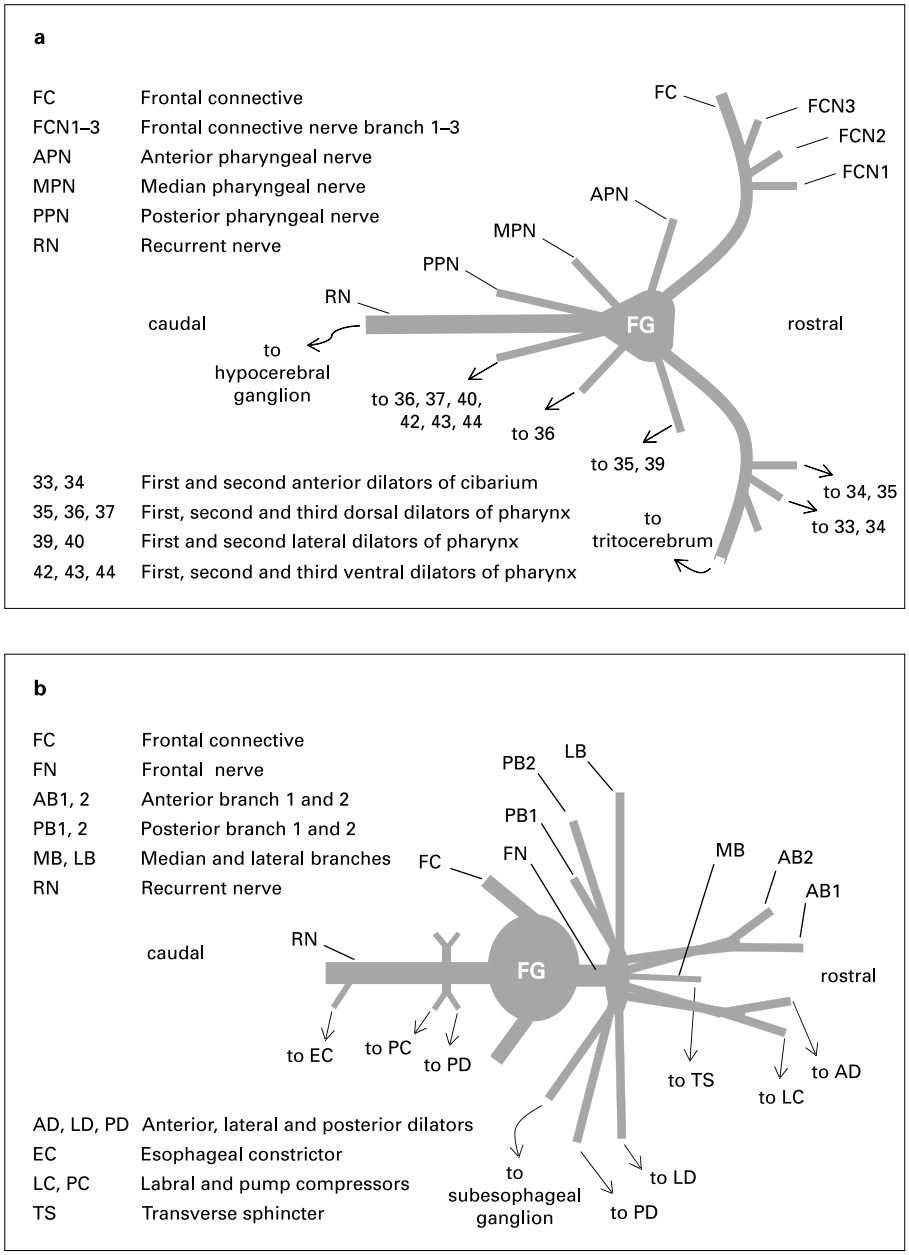

Fig. 2. A schematic drawing of the FG of the locust (**a**) and *Manduca* (**b**). In each panel, the upper half shows the ganglion nerves and the lower half the muscles they innervate. Caudal and rostral sides are also indicated.

cade later, in *The Neurobiology of an Insect Brain,* Burrows [2] made the following comment: '... virtually nothing is known of their (the stomatogastric or stomodeal ganglia) physiological actions, in stark contrast to their counterparts in crustacea.'

In spite of the above statements, the function of the insect stomatogastric nervous system has been the focus of many studies for more than 100 years [61, 62]. Most of these studies, however, examined the effects of ablating the FG on the subsequent behavior and development of the insect [10, and references therein].

Roussel [63] reported a role for the FG in the control of cardiac rhythm in *Locusta migratoria*. These findings were repeated in the sweet potato hornworm, *Agrius convolvuli*, where the FG was also found to control heartbeat [64, 65]. This control is exerted via a pair of anterior cardiac nerves that branch off the FG visceral nerve to innervate the dorsal vessel.

Most previous results have indicated that the FG is instrumental in the processes of growth, water balance and molting [e.g. 66–80]. These findings have been partially supported by recent neurophysiological work which has concentrated on the role of the FG in the control of two fundamental behaviors in the life of insects, feeding and molting [45–50].

The Role of the Frontal Ganglion in the Control of Feeding-Related Behavior

Frontal ganglionectomy caused a decrease in feeding activity and food intake in *S. gregaria* [30, 67, 69], *L. migratoria* [72], *Gryllus bimaculatus* [68] and *P. americana* [73, 81, 82]. Food was reported to accumulate in the foregut, and fecal output was markedly reduced [30, and references therein]. Similar results were reported in Lepidoptera, including adult *Heliothis zea* [78] and *M. sexta* larvae [79]. Overall, from these multiple studies, one can deduce that the insect FG is instrumental in passing food through the foregut and in crop emptying.

The larval *Manduca* gut is constantly active. The FG neurons were found to innervate all the larval foregut muscles and the ganglion was reported to be both necessary and sufficient for producing the motor patterns of the foregut [48]. Two types of rhythmic foregut movements and, accordingly, two FG rhythmic motor patterns were described in fifth-instar larvae. The first are posteriorly directed waves of foregut peristalsis, which are generated by phase shifts between rhythmic bursts of activity in anterior and posterior constrictor muscles (fig. 3a). The second type of pattern is characterized by synchronous constriction of muscles along the entire esophageal region. The latter motor pattern was reported to be correlated with accumulation of food within the crop [48]. Presumably it serves to pack the food particles and prevent food from reverting from the crop. In both the described rhythmic patterns, buccal constrictor activity preceded esophageal constrictors.

In contrast to the larvae of most Lepidoptera, locusts are generalist feeders, consuming a wide variety of foods of different composition and form. This wide range, together with the more complex morphological structure of their foregut (in comparison to the *Manduca* larvae), has led to more complex foregut peristaltic behavior in locusts. Rhythmic activity is not always demonstrated by the locust foregut muscles [46]. Most interestingly, as is the case in the *Manduca* larvae, the intact locust shows two types of FG motor patterns [46]. However, in the locust only one of these patterns could be related to feeding behavior: a rhythmic motor pattern, consisting of bursts of action potentials recorded on the different motor nerves, which is consistent with a rostrum-to-caudal peristalsis wave in foregut muscles (fig. 3b). This 'food passage' behavior can be recorded from the FG nerves in association with the beginning of a feeding bout. The rhythm increases in cycle frequency as food accumulates in the foregut and crop, and practically stops as soon as the locust gut is full [46]. Between meals, the FG pattern is often totally inhibited; in other cases, it demonstrates a second pattern, which is characterized by full synchronization between bursts of action potentials recorded on the different motor nerves, and both between the FG and the ongoing ventilation motor pattern of the locust [46]. It thus appears that gut movements may also participate in ventilation, probably as a means to help with hemolymph circulation.

Unlike the case of the larval moth, and more similar to the locust, the adult *Manduca* cibarial pump motor program is only displayed during feeding [49]. Unless the moth is feeding, the muscles of the cibarial pump are silent. The FG activity pattern that generates rhythmic pumping movements in the moth's cibarium is initiated by chemical stimulus to the proboscis of the moth.

Much work is still needed in order to elucidate the role of the FG neural networks in insect feeding-related behavior. As is apparent from the rather limited neurophysiological data available, new insights could be gained by comparing the stomatogastric nervous system in the locust and moth preparations and in the different developmental stages of the holometabola.

The Role of the Frontal Ganglion in the Control of Molt-Related Behavior

As already mentioned, in addition to feeding, the foregut and insect stomatogastric nervous system play a critical role in at least one other aspect of insect life: the molt. A molting insect displays a stereotypical set of behaviors that culminate in the shedding of the old cuticle at ecdysis. Ecdysial behavior has been extensively characterized in crickets [83, 84], locusts [85–87] and moths [88–91]. In all these insects, as the molt approaches, the insect ceases all feeding-related activity and searches for a suitable site for ecdysis. For example, both *S. gregaria* and *Manduca* larvae stop feeding and become quiescent approximately 24–36 h before ecdysis [50, 87, 92]. This is part of a preparatory or pre-ecdysis phase, which includes motor patterns that are aimed at loosening and eventually splitting the old cuticle. Next, the insect extracts itself from its old cuticle, followed by an expansion period, during which the new cuticle is stretched and shaped and the wings are expanded and folded.

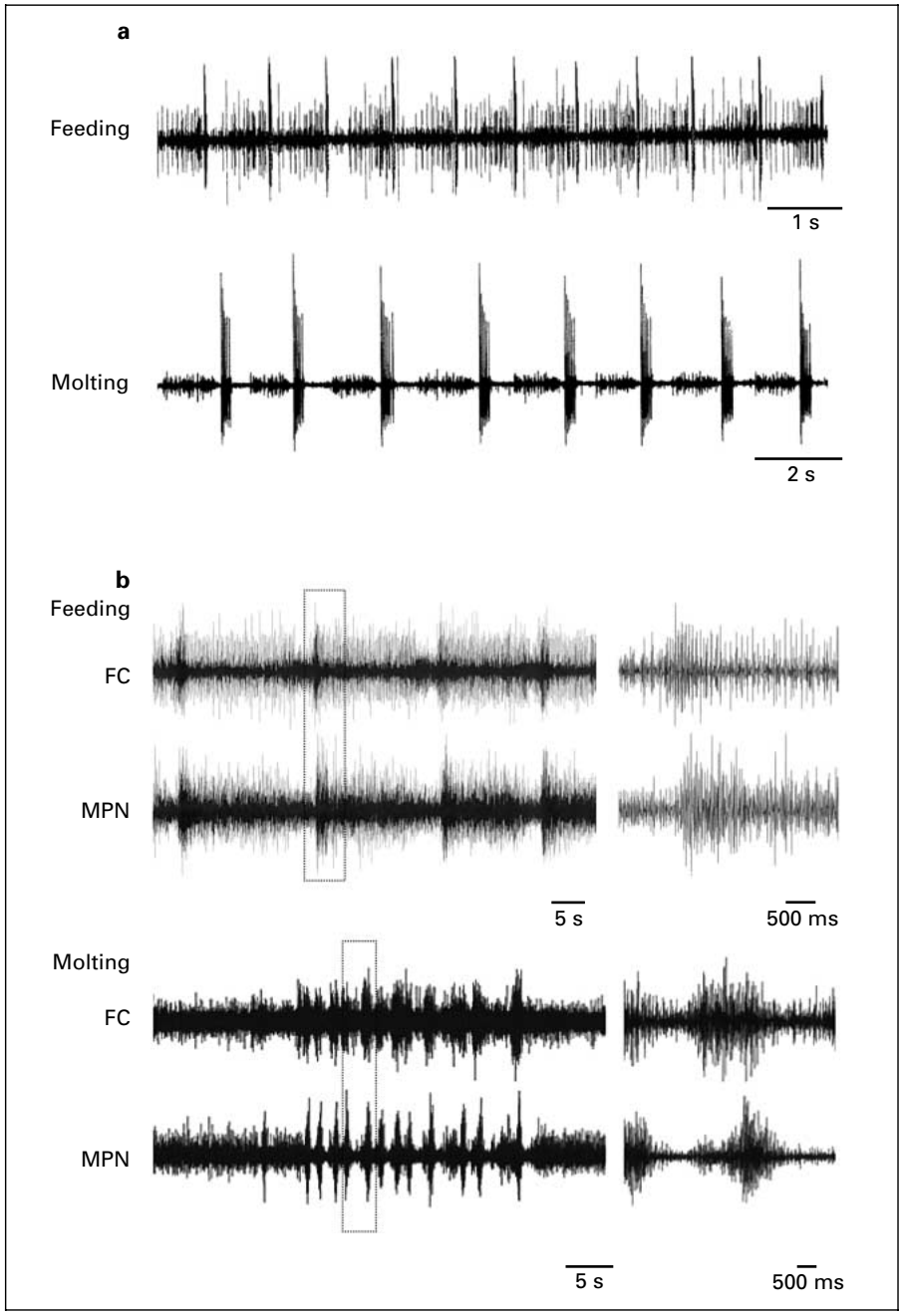

Fig. 3. FG feeding- and molting-related motor patterns in *Manduca* (**a**) and the locust *S. gregaria* (**b**). **a** The motor pattern recorded from the adult moth cibarial and our swallowing pump compressor (large units) and dilator muscles during feeding. Data are courtesy of CI Miles. **b** Simultaneous extracellular recordings of locust FG frontal connective (FC) and median pharyngeal nerves (MPN) during feeding and air swallowing behavior (lower trace). The boxed areas are shown in faster sweep speed on the right.

Hence, there are two stages during ecdysis in which the insect needs to exert pressure on the body wall [88]. The first is during rupture of the old cuticle, the second when expanding the new cuticle and wings after emergence. The principal mechanism for doing this is by filling the gut with air. De Bellesme [93] was the first to show that the pronounced enlargement of freshly emerged dragonflies was accomplished by internal air pressure built up in the digestive tract [18]. Since then, air swallowing during ecdysis has been reported in a number of different insects [49, 76, 85, 88, 94]. The FG was reported to be important for this behavior in several species [14, 15, 49, 50, 66, 71, 75–77].

Hughes [74, 75] reported that the success of the imaginal ecdysis of the desert locust depends on inflation of the gut with air. The dynamics of the air-swallowing motor program during the imaginal ecdysis was monitored by electromyogram (EMG) recordings made from foregut dilator (extrinsic) muscles [74, 75]. Elliot [95] accompanied the EMG recordings during the course of the locust molt by intracellular recordings from a small number of motor neurons in the FG, supporting the fact that the ganglion is indeed the source of the molt-related foregut motor pattern [95]. Frontal ganglionectomy abolished air swallowing immediately [74]. In the cricket, Carlson and O'Gara [76] have also described the FG's exclusive control over air-swallowing motor patterns and its importance for a successful molt. Ayali et al. (unpublished results) have recently confirmed these findings by testing the effects of ablating the ganglion of fifth-instar larval locusts, 48 h before the imaginal molt, on the probability of successful ecdysis. One hundred percent of the experimental animals (n = 8) failed to escape the old cuticle and died during the molt. In contrast, all sham-operated animals (n = 8) molted successfully.

Zilberstein and Ayali [46] report a strong interaction between the locust FG and ventilation pattern generator circuits during ecdysis. Throughout the molt process, the FG and ventilatory patterns are totally synchronized, except for the very short period when air-swallowing behavior is activated. During air swallowing, a different pattern emerges that resembles the feeding-related pattern in many aspects (fig. 3b). This uncoupling of the ventilation and FG rhythms could be mimicked by experimental manipulation [46].

Air swallowing was also reported in Lepidoptera. Bell [77] suggested that the FG also plays a role in *M. sexta* eclosion, i.e. ecdysis to the adult stage. The FG was involved in swallowing air at the time of eclosion; frontal ganglionectomy abolished air swallowing immediately, leading to defects in eclosion and in expansion of the wings.

Recent work on *M. sexta* has revealed that the FG plays a critical role in the successful completion of both larval [50] and adult molts [49]. At both stages, the FG controls a foregut motor pattern that is used to remove molting fluids from the space between the old and new cuticle prior to ecdysis. Cornell and Pan [96] were the first to suggest that the gut played a role in the removal of molting fluids. At adult ecdysis, or eclosion, removing the FG resulted in difficulty or failure to shed the old cuticle [49]. The FG is activated about 6 h before the adult moth emerges from the pupal case. The crop initially fills with molting fluid, then air. After eclosion, as the moth hangs in a position to expand its wings, the FG is again activated, producing a distinct air-swallowing motor pattern that lasts about 90 s (fig. 3a). During this period, the wings visibly expand. Miles and Booker [49] report that the few ganglionectomized individuals that successfully emerged from the pupal cuticle were unable to expand their wings. The motor pattern recorded from the FG at the time of eclosion is similar to that displayed during feeding (fig. 3) [49].

It is interesting to note that in aquatic arthropods (Crustaceans) the stomatogastric nervous system probably plays an equally important role in swallowing fluids in order to split the old cuticle. This has as yet not been investigated, though it was implied in many reports [e.g. 97–100])

Stomatogastric Neural Circuits

Electrophysiological studies of the stomatogastric nervous system of insects have been very rare. Möhl [32] was the first to conduct investigations of neural activity in the insect system. His work on *A. domesticus* was followed by Hertel [101], who studied the stomatogastric nervous system of *P. americana*. The latter described spontaneous nervous activity in the caudal parts of the system, most of which could be traced to the FG [101, 102]. As was also later confirmed by Pandey and Habibulla [103], spontaneous neuronal activity could be recorded from an isolated FG in vitro. Hertel and Penzlin [104] demonstrated spontaneous rhythmic burst activity in the stomatogastric nervous system of *P. americana* and *Blaberus craniifer.* Again, these authors suggested that the rhythm is generated in the FG and from there disseminates throughout the stomatogastric system. However, no physiological function could be assigned to this activity [10].

More recent reports for both *M. sexta* [48] and *S. gregaria* [45] have confirmed the early work and established the presence of a central pattern-generating circuit in the insect FG. The larval *Manduca* ganglion was spontaneously active and produced a bursting firing pattern in the total absence of descending or sensory inputs [48] (fig. 4a). Interestingly, the pattern demonstrated by an in vitro fully isolated preparation resembled that recorded prior to its isolation (characterized by phase shifts between rhythmic bursts recorded from the different efferent nerves, or a fully synchronized bursting pattern (Miles, pers. commun.).

A completely isolated in vitro locust FG also generated a robust and consistent spontaneous rhythmic motor pattern that could last for many hours (fig. 4b) [45]. The in

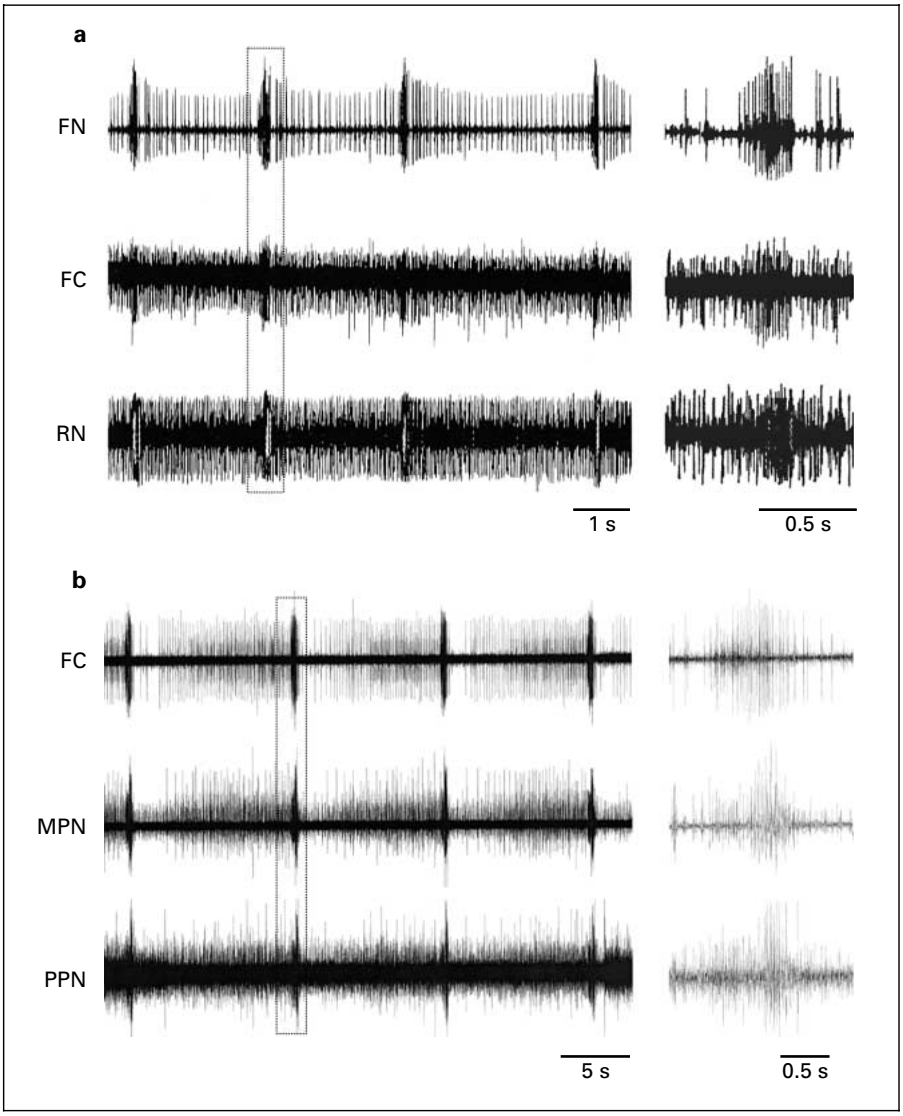

Fig. 4. a Simultaneous recordings from the frontal nerve (FN), recurrent nerve (RN), and frontal connective (FC) in a fully isolated FG dissected from a larvae *M. sexta*. **b** Simultaneous recordings from the frontal connective (FC), median and posterior pharyngeal nerves (MPN and PPN, respectively) in a fully isolated locust FG in vitro. The panels on the right show 1 burst of activity played at a higher sweep speed to reveal phase relations between different members of the FG central pattern generator.

vitro pattern was found to be independent of the donor locust's physiological or developmental stage. It was characterized by multi-unit bursts of action potentials that could be recorded from the various FG motor nerves. Ayali et al. [45] have defined the locust FG rhythmic pattern in vitro as fictive feeding-related or 'food passage' behavior, based on analyzing the temporal delineation of bursts of action potentials recorded on the different motor nerves. Considering the muscles innervated by these nerves, the pattern was consistent with a rostrum-to-caudal peristalsis wave in foregut muscles. Interestingly, in another closely related Orthopteran, the cricket *Teleogryllus oceanicus*, an isolated FG in vitro was reported to generate rhythmic activity only for 1 h [76]. Furthermore, in vitro rhythmic activity was exhibited only if the ganglion was dissected out of a molting insect and was characterized by a normal air-swallowing frequency [76].

The locust FG contains around 100 neurons [105], in between the numbers reported for the ganglion of other Orthoptera, such as the cockroach (circa 80 neurons [106]) and the cricket (150 neurons [21]). Are all these neurons members of the pattern-generating network(s)? In most of the central pattern-generating systems investigated, the pattern-generating circuit consists of interneurons, though in some preparations the motor neurons

themselves participate in generating the rhythm (e.g. the stomatogastric nervous system of Crustacea [107, and references therein]). Kirby et al. [21] suggest that no more than 25% of the neurons in the FG of *A. domesticus* are interneurons. Aubele and Klemm [19] described 19 neurons located in the locust FG that send their axons to innervate foregut muscles via the frontal connectives and their branches. Similar numbers of small-size neurons (motor neurons) were also reported by Elliott [95]. The rather limited number of rhythmic units included in FG nerve recordings [45], and some preliminary intracellular survey of the ganglion neurons in which the majority of cells proved to be either silent or tonically active (Ayali, unpublished results), are both consistent with the idea that only a relatively small number of the ganglion neurons take part in the FG rhythmic motor pattern. Hertel and Penzlin [104] suggested that the FG spontaneous rhythmic activity in *P. americana* is generated within the ganglion's neuropil in a pair of neurons whose cell bodies are located outside the ganglion, in the protocerebrum. These neurons send their axons to the FG via the nervous connectives.

Our knowledge of the neuronal characteristics or the cellular properties of members of the insect FG neural circuit is limited. In *Manduca*, only a few of the 35 FG neurons have been explored, using intracellular pipette recordings [48–50]. A number of motor neurons have been identified by correlating their spiking activity with specific muscle excitatory junction potentials. Nothing, however, is known on the synaptic connections between the network members and on their role in generating the rhythmic pattern. The locust FG has proved to be a challenging system for intracellular analysis [45, 95]. However, the limited intracellular recordings made from locust ganglion neurons demonstrate the presence of some properties which are considered to be important for central pattern generation, such as bursting, plateau potentials and post-inhibitory rebound. Recently, Shefi et al. [108] developed a culture preparation of dissociated locust FG neurons. Intracellular recordings from unidentified isolated FG neurons in culture just as they started to regenerate their neuronal processes, also revealed some of the neurons' endogenous properties, such as spontaneous firing and post-inhibitory rebound (Ayali, unpublished results). Extracellular recordings from two-dimensional networks in cultures of FG neurons revealed rhythmic bursting (Ayali et al., unpublished results).

Some information on the synaptic properties of the FG central pattern generator networks can be gained from sporadic evidence regarding effects of cholinergic agonists and antagonists on the insect FG's electrical activity ([104, 109, 110]; Ayali and Dekel, unpublished results). Acetylcholine is an important transmitter in insects. It has also been reported to be a neurotransmitter in the stomatogastric nervous system of the earthworm [25] as well as in Crustaceans [111–113]. Acetylcholine and nicotine showed dose-dependent stimulation of the FG rhythm in *P. americana* [104]. Hertel et al. [110] reported on dose-dependent effects of different organophosphorous insecticides on spontaneous burst frequency of isolated ganglia. Increasing concentrations resulted in shortening of the inter-burst intervals, up to total loss of rhythmicity. Similar results were obtained in *S. gregaria* by bath application of the acetylcholinesterase inhibitor paraoxon to a rhythmically active, isolated FG in vitro (fig. 5; Ayali and Dekel, unpublished results). Penzlin [10] reviewed evidence of high acetylcholinesterase activity in the neuropil of the FG in *P. americana*. However, the cockroach stomatogastric nervous system ganglion cells could not be histochemically stained.

Thus, although ample evidence suggests an important role for acetylcholine in the insect FG, as yet the data do not distinguish between cholinergic synaptic transmission and cholinergic modulation.

Neurosecretion and Neuromodulation in the Stomatogastric Nervous System

The way by which the function of the nervous system is modified to allow an animal the behavioral plasticity needed to adapt to the changing demands of its environment is a fundamental question in neurobiology. Substantial progress has been made by studying rhythmic behaviors and the central pattern generator circuits that generate them [114, 115]. It is now clear that the nervous system can alter the properties of central pattern generators, via both descending as well as sensory inputs, to elicit many different motor patterns [e.g. 22, 116–121]. Thus the motor patterns of the insect foregut are expected to be multiple and complex, and the FG central pattern generator controlling these motor patterns is expected to generate various motor outputs, depending on the animal's physiological and behavioral state.

Neuromodulation of the Frontal Ganglion Central Pattern Generator

As already described, the insect stomatogastric nervous system plays a major role in two behavioral contexts: feeding and molting. Both behaviors (especially the latter)

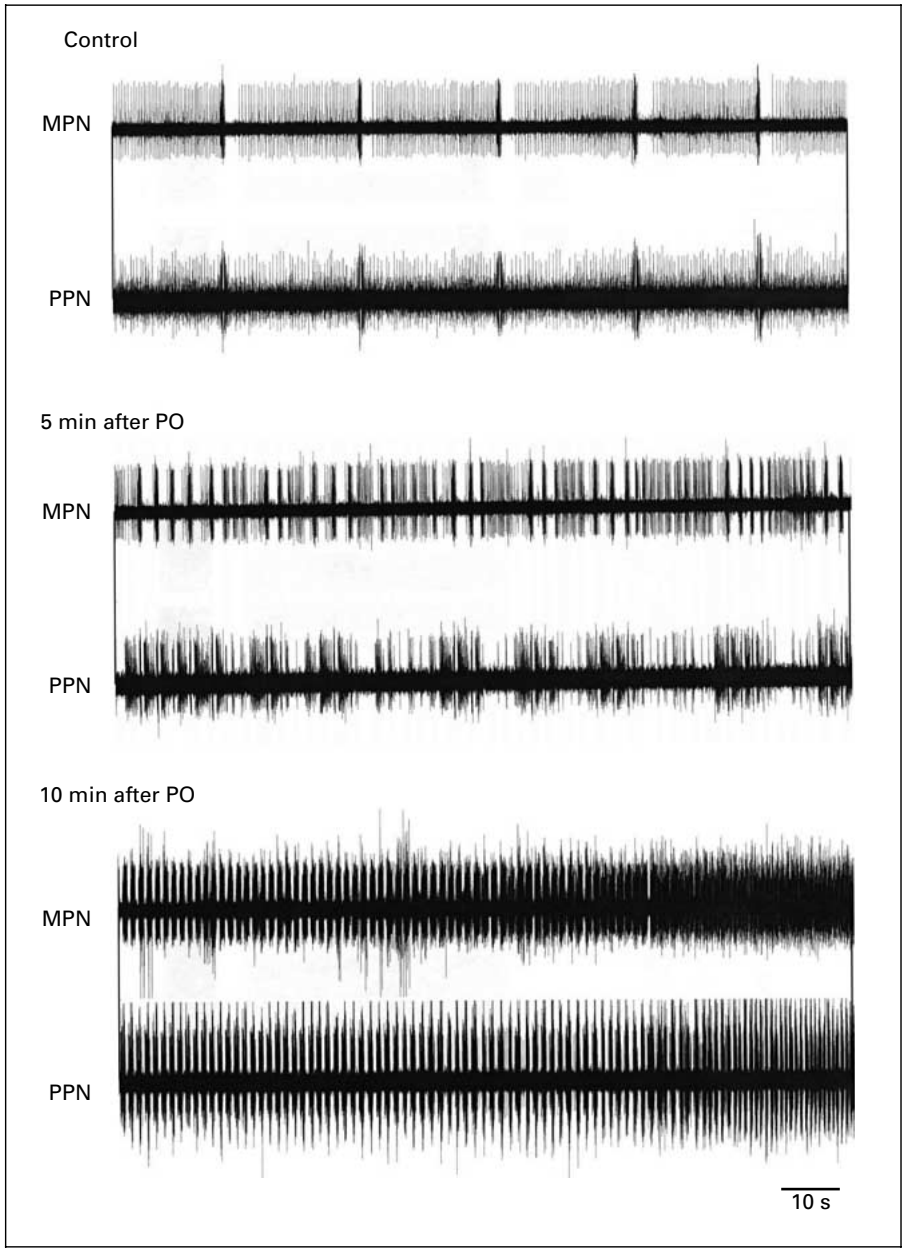

Fig. 5. Simultaneous recordings from the median and posterior pharyngeal nerves (MPN and PPN, respectively) in a fully isolated locust FG in vitro. Data show the FG rhythm in control conditions, 5 and 10 min after bath application of 1m*M* of paraoxon (PO). The organophosphorous compound PO inhibits the enzyme acetylcholinesterase irreversibly.

are made up of a complex set of motor patterns that need to be carefully coordinated and controlled.

Volumetric feedback from the gut has been suggested in several preparations and undoubtedly takes part in the control of the stomatogastric nervous system and feeding-related motor output. Sensory information mediated via stretch receptors from the gut wall has been shown to be instrumental in the control of feeding in the fly [122–125]. Volumetric feedback from the crop and hindgut was reported to interact in the regulation of meal size in crickets [126] and locusts [127]. Clarke and Langley [13] reported that in *L. migratoria,* the FG forms a link in the passage of nervous impulses originating from the stretch receptors of the pharynx and passing via the posterior pharyngeal nerve, FG and frontal connectives to the brain. Consistent with these early reports [see also 20], Zilberstein and Ayali [46] found that the amount of food present in the locust gut modulates the frequency of the

FG rhythm. The FG and foregut feeding-related rhythmic patterns were totally inhibited when the entire gut seemed replete with food [46]. In *M. sexta* the pattern of foregut activity has also been shown to vary with the amount of food present in the foregut and crop [48].

The ascending signals of gut sense organs (e.g. stretch receptors) could either produce inhibition directly or generate central inhibition [79]. Miles and Booker [48] showed that isolating the FG of *Manduca* larvae from the brain results in alteration of the FG rhythmic pattern. As reported for other insect central pattern generators [128], inputs from the brain were shown to have an inhibitory effect on the locust ganglion in an in vitro FG-brain preparation. The locust FG rhythmic pattern emerged only after severing the frontal connectives coming from the brain [46]. Other neuronal inputs to the FG have also been suggested. As already mentioned, Zilberstein and Ayali [47] reported on interactions between the FG and the thoracic ventilation central pattern generator, which are mediated via the frontal connectives. Backfilling the frontal connectives resulted in staining several neurons in the subesophageal ganglion and in thoracic ganglia all the way to the metathoracic ganglion, in which a single neural cell body was stained (Zilberstein and Ayali, unpublished finding). The metathoracic ganglion is where the locust ventilation central pattern generator resides [129].

Feeding-related motor patterns in the stomatogastric nervous system could also be controlled by humoral factors or regulatory neurohormones released into the circulation. These can be similar or different to those acting in classic synaptic transmission in the above-mentioned neuronal pathways. Release of humoral factors that play a role in cessation of locust feeding and involvement of chemoreceptors of the foregut was already suggested by Bernays and Chapman [130]. Ayali et al. [45] reported that application of hemolymph collected from locusts with a very full gut and crop to an isolated FG in vitro inhibits an ongoing rhythm.

Chemical modulation of FG motor patterns may also be working via localized release of neuromodulatory substances into the ganglion neuropil. In their electron microscopic and immunohistochemical study of the FG of *P. americana*, Ude et al. [131] reported a high content of neurosecretory material limited to the FG neuropil. These authors suggested that the autonomous control of the FG over vegetative function is influenced (i.e. modulated) by extrinsic neurosecretory cells establishing contact with intrinsic neurons within the ganglion. In *A. domesticus*, Kirby et al. [21] described neurons with cell bodies in the brain's median neurosecretory area that send their axons to the FG, and suggested that these neurons are the source of the neurosecretory material described by many authors in the FG [see references in 21].

Two candidates for feeding-related FG neuromodulation will be discussed below: first, peptides of the allatostatin peptidergic family, and second, the biogenic monoamine octopamine.

Allatostatins are a large group of neuropeptides identified and localized by immunocytochemical means in many different insect species [132–137]. Besides their original physiological role as inhibitors of juvenile hormone production, members of this rich family of peptides have been reported to demonstrate inhibition of myotropic activity [138, and references therein]. Maestro et al. [134] have observed allatostatin-immunoreactive neurons in the tritocerebrum of *Blattella germanica*, with axons projecting and branching into the FG. This is of great interest in the light of recent data by Zilberstein and Ayali [47], who reported modulatory effects of allatostatin on the rhythmic output of an isolated locust FG in vitro. Furthermore, as described in the next section, FG cells that contain allatostatin immunoreactivity have been reported in various insects. The question whether the FG network is auto-modulated by neurosecreting neurons from within the ganglion is very intriguing.

Octopamine is an important modulator of neural function and behavior in insects. Octopamine modulation of insect rhythmic behavior has been repeatedly described [e.g. 139, 140, 141]. In relation to feeding behavior, in a recent study Miles and Booker [142] suggested that the dramatic decline in foregut activity demonstrated by *M. sexta* larvae parasitized by the braconid wasp *Cotesia congregata* was due to a sharp increase in hemolymph octopamine. Octopamine may also be a modulator of feeding and gut motor patterns in normal healthy larval moths.

In accordance with the above report, application of octopamine to an isolated locust FG in vitro disrupted all rhythmic activity [47]. Braunig [143] reported on subesophageal, octopaminergic dorsal unpaired median neurons which project into the FG. The ganglion is reached via the frontal connectives. Additional axon collaterals project into the numerous side branches of the nervous corporis cardiaci III, which innervate the hypocerebral ganglion and several pharyngeal dilator muscles. Thus one can speculate that octopamine, which is often correlated with arousal state in insects [144–146] and plays an important role in locust flight [145, 147, 148], will work to inhibit feeding-related behavior during demanding and stressful physiological states.

It is interesting to note that a role in the regulation of the stomatogastric nervous system was suggested for another biogenic amine, histamine. Horner et al. [149] reported that the histamine-immunoreactive terminals in the neuropil of the FG of *G. bimaculatus* have a neurosecretory appearance. This observation was also confirmed in the cockroach *Leucophaea maderae* [150].

The same mechanisms that act to shape feeding-related motor patterns in the insect stomatogastric nervous system also apply to the molt. Hughes [75] suggested a role for volumetric feedback in regulating the air-swallowing motor program during ecdysis. This was confirmed by Zilberstein and Ayali [46], who punctured the fully inflated gut of an adult locust when it was expanding its wings just as it fully emerged. A characteristic FG air swallowing rhythm was instantaneously initiated to compensate for loss of air pressure in the gut.

A number of peptide hormones are currently believed to be involved in the control of insect ecdysis behavior [151]. Carlson and O'Gara [76] reported that the cricket FG could generate spontaneous activity in vitro only if isolated from near-molt, ecdysing, or expanding insects. This state of 'activation' wore out within 1 h, during which the ganglion generated an air-swallowing pattern. In *Manduca* there is also evidence for foregut and FG modulation during the molt: hemolymph collected from molting larvae and applied to a larval FG-foregut preparation altered the ongoing feeding motor pattern to resemble that observed in molting larvae. A rhythmic motor pattern that resembles air swallowing could be generated in isolated heads of animals 24–30 h from eclosion by application of eclosion hormone (EH) [49]. Interestingly, in the locust, hemolymph collected from non-feeding premolt larvae inhibited FG rhythmic activity [45], and Zilberstein and Ayali [47] have reported that eclosion hormone transiently inhibited the FG rhythmic pattern in vitro. These different and somewhat contradictory observations can be explained by considering the different and complex effects of the various insect ecdysis-related peptides [151–154], and by suggesting that exact timing is crucial for the experimental manipulations. Zitnan and Adams [155] suggest that the initiation of pre-ecdysis behavior and the transition to ecdysis are regulated by stimulatory and inhibitory factors released within the central nervous system (CNS) after the initial actions of pre-ecdysis-triggering and ecdysis-triggering hormones. These factors, including EH, which is released both centrally into the brain and CNS and into the circulation, set the temporal organization of ecdysis behavior and the progression from one phase to the next. Thus, to activate each subunit of the molt set of programmed behaviors, specific neural elements, including the FG neural network(s), are targeted at precise time points.

According to the current scheme, crustacean cardioactive peptide (CCAP) plays a role in maintaining the ecdysis motor program in insects [151]. Bestman and Booker (pers. commun.) have observed that CCAP generated increased motility and, in many cases, peristalsis in the molting larvae foregut. In locusts, Zilbertstein and Ayali [47] demonstrated a modulatory effect of CCAP on the rhythmic pattern of the FG: a dose-dependent excitatory effect was obtained by application of CCAP to an isolated locust ganglion in vitro. In accordance with these reports, Dircksen and Homberg [156] observed CCAP immunoreactivity in locust brain neurons that gave rise to extensive arborization within the FG neuropil as well as in a FG neuron.

The Insect Stomatogastric Nervous System and Frontal Ganglion as a Source of Neurosecretion

In contrast to the rather limited studies on other aspects of insect stomatogastric nervous system physiology, very rich literature is available on neuropeptides and other neurosecretory substances in cells of the stomatogastric system. Nevertheless, early reports regarding the presence of neurosecretory cells in the FG were somewhat controversial [10, and references therein].

Peptide-producing neurosecretory cells have been described in the FG of a number of lepidopterous insect species. Bounhiol et al. [157] described two such cells in *Bombyx mori*. This was supported by similar findings in *Manduca* [35, 36] and *Diatraea grandiosella* [158]. No specific role was suggested for these cells. Different degrees of activity in diapausing and non-diaposing pupae indicate a close relationship to developmental processes. Recently Duve et al. [135] reported on two pairs of large FG cells in *Helicoverpa armigera* that demonstrate allatostatin immunoreactivity, with one of the pairs showing colocalization with allatotropin [see also 137]. The axons of all four cells project to the brain via the frontal connectives and to the foregut via the recurrent nerve [136]. It is thus suggested that peptidergic neurons in the FG play a major role in regulating foregut motility ([135], see also previous section).

Penzlin [10] gave a thorough report of evidence for the presence of unidentified neurosecretory cells in the stomatogastric nervous system of different Orthoptera (mainly *Periplaneta* and locusts). A considerable amount of data regarding neurosecretory (mostly peptidergic) cells in the FG has accumulated since that review article was

published. Cell bodies that show immunoreactivity to a myotropic neuropeptide, Lom-AG myotropin I were detected in the FG of *L. migratoria* [159]. Locustatachykinin I (another myotropic neuropeptide) immunoreactivity was also found in cells of the FG of the locust [160, 161]. Myoshi and Endo [162] investigated the FMRFamide-containing peptidergic neurons in the stomatogastric nervous system of the American cockroach by immunoelectron microscopy. Immunoreactive cell bodies were located in both the ingluvial and frontal ganglia.

Luffy and Dorn [163] reported the presence of seven pairs of serotonergic perikarya in the FG of *Carausius morosus*. These authors also reported a strong excitatory effect of 5-HT on the isolated midgut. This was also observed in *Teleogryllus* by Cooper and He [164]. In another cricket, *G. bimaculatus*, serotonin and other major biogenic amines (octopamine, dopamine) were reported to be synthesized and metabolized in the FG [165], where the amount of serotonin was found to be highest.

Thus insect FG neurons are an important source of neurosecretion. The neurosecretory material from the FG can modulate muscles of the alimentary canal, thus providing an additional route for the stomatogastric nervous system control of gut motor patterns in feeding- and molting-related behavior.

Finally, there is even evidence for the presence of insulin in the FG of *M. sexta* [166], though no specific role for this has yet been suggested.

Concluding Remarks

Studies of identified neurons in insects have provided unsurpassed data on neural mechanisms of behavior [4]. In order for the insect FG and stomatogastric preparation to reach its full potential, much more work is needed in identifying and characterizing members of the FG central pattern generator in *Manduca* and the locust (for which much information is already available), as well as in other insect preparations.

A comparative approach in future research is important in order to elucidate the physiological significance of the FG and stomatogastric nervous system in insect behavior. More specifically, comparative studies across different insect species as well as different developmental stages are needed. The FG of Lepidoptera consists of circa 35 neurons, while that of the Orthopteran insects features three times this number or even more. Comparing the neuronal composition of the FG (and other stomatogastric ganglia) between these groups will shed light on the role of the different components in generating and controlling behavior. Comparing the stomatogastric nervous system of the larval and adult stages in holometabolous insects is also an important question with implications for developmental neuroscience. Since the same set of neurons drive both the larval foregut and the adult cibarial pump, as neurons are neither added nor removed from the FG during adult development [49], it will be interesting to determine how the functions of the larval neurons change during adult development.

Finally, the question of motor program selection or the choice of motor pattern is fundamental in the study of the nervous system [167]. Hence, much of the appeal of the insect stomatogastric nervous system as a future neurobiological model system arises from its important dual role in feeding- and molting-related behavior. The specific motor output of defined neural circuits within the system must be generated at the appropriate time, in full coordination with other complex behavioral patterns. Hence, neuromodulation and the interaction between neural centers are fundamental concepts in understanding this system. Following the investigations described herein, comparing the feeding- and molting-related FG motor patterns, the way they are generated within the stomatogastric nervous system and the neural and chemical factors that shape and control them, is the mission we are currently engaged in.

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Review

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Molecular Mechanisms for *Drosophila* Neuronetwork Formation

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Key Words

Drosophila · Axon · Dendrites · Guidance · Branching · Targeting

Abstract

Understanding the establishment of functional neuronetworks is one of the frontiers of developmental neurobiology. The use of axons and dendrites from *Drosophila* nervous system as a model allows the identification of molecular mechanisms which give neurons the ability to guide their processes en route to connect precisely with their partners. By focusing on selected *Drosophila* model systems, we discuss the recent advances in our understanding of the molecular mechanisms regulating guidance, branching and targeting of axons and dendrites required for the establishment of a functional neuronetwork.

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Introduction

One of the essential requirements for constructing a functional neuronetwork is correct matchmaking between pre- and post-synaptic partners. To achieve this, a neuron seeks its partners while extending multiple processes, typically a single axon and multiple dendrites, often over a rather long distance. This correct matchmaking requires that the neuron follows a succession of steps such as growth, guidance and branching of processes as well as their correct targeting and synaptogenesis. Understanding the construction of a functional neuronetwork implies understanding the molecular mechanisms of axonal and dendritic growth, pathfinding and target recognition.

Drosophila has been used for decades to elucidate various developmental processes through its powerful genetic tools. The analyses of mutants and the possibility to conduct genetic screening for particular phenotypes have been applied to decipher the molecular mechanisms leading to a functional neuronetwork. The constant development of genetic and cell visualization tools has allowed *Drosophila* neurobiologists to generalize the use of various neuronal models at different developmental stages. It also allowed the conduction of analyses at the single-cell level, as well as single-cell restricted genetic manipulations. Several neuronal models have been chosen for their best adequacy to study a particular step in the formation of functional neuronetwork. The study of neuronetwork development in such models highlights some constants in the programming of neuron connectivity. In this review, we discuss the molecular mechanisms controlling several important steps a neuron has to take to meet its correct partners and start long-term relationships with them.

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Guidance

Checkpoint Decision at the CNS Midline

Once a neuron is born, it starts to grow processes that will become the axon and dendrites. The mechanisms that regulate the polarity of the neuron as well as where the axon and dendrites grow are yet to be understood, but it appears to be a controlled process. To grow over a long distance, axons rely on the interpretation of both long-range and short-range cues provided by the environment. They follow trajectories that are staked out with multiple checkpoints, dividing axon guidance into a series of decision-making events [1].

The CNS of bilateral animals is physically divided by the midline. To ensure coordination between the two sides of the brain, communication has to be established and also regulated. To this end, specific neurons send their processes, either axon or dendrites, across the midline, creating communication lines displayed as commissures. Therefore, CNS neurons have two choices regarding their projections, remaining ipsilateral and avoiding the midline or projecting contralaterally and going through the midline. The midline, with its yes or no question, is a perfect paradigm for studying how neurons make a directionality choice at a checkpoint.

In *Drosophila*, the embryonic abdominal CNS is constituted by a repetition of identical segments (segments A2–A7), each of which is composed of mirror-image hemisegments. Each abdominal hemisegment is reported to contain 342 neurons [2], including 34 that are motoneurons exiting the CNS to innervate 30 abdominal muscles [2, 3]. The position of neurons, as well as the directionality of their projections, both axons and dendrites, and their targets are highly stereotypic, conserved from one hemisegment to another and from one animal to another, making the ventral nerve cord an ideal model for genetic and cellular analysis. The midline is materialized by the presence of three essential glial cells, hereafter referred to as midline glia cells per segment. Several markers expressed on neuron projections allow the visualization of the wild-type stereotypic organization of the embryonic ventral nerve cord. Among them, the antibody BP102 labels specifically the whole embryonic neuropile revealing a 'ladder-like' structure with longitudinal connectives connected by a pair of anterior and posterior commissures in each segment [4]. As for the question of midline, crossing the embryonic nervous system can be simplified as a model in two dimensions with ipsilateral and contralateral neuropiles linked by commissures.

BP102 antibody has been used widely to identify mutations disrupting the general organization of the neuropile. These mutations can be classified into two main groups. The first group includes mutations for which the commissures appear thickened by an excess of fibers crossing the midline. The second group consists of mutations for which the commissures look thinner due to the reduction in the number of fibers crossing the midline. These opposite phenotypes suggested a model in which excess crossing is due to the lack of activation of a pathway triggering repulsion, and reduced crossing is due to the absence of activation of an attraction pathway [1]. Several molecules that generate this disorganization of midline crossing have been identified and their function characterized. They exemplify how neurons rely on their extracellular environment for their guidance, while emphasizing the importance of midline glia cells as an organizing center of the CNS projections [5].

Midline glia cells secrete ligands that activate receptors localized on the surface of neurons, triggering either attraction or repulsion. Neuronal response depends on the receptor they activate, leading the neuronal processes to cross the midline or not.

NetrinA and NetrinB, secreted by midline glia, interact with their receptors, Frazzled and Unc5 [6–9]. When *frazzled* is mutated, commissures appear greatly reduced and the longitudinal tracts are periodically interrupted [7]. Conversely, when *Unc5* is overexpressed in all neurons, processes fail to cross the midline [9]. Both responses are dependent on Netrins, but neither receptor requires the other for its function [9]. Therefore, it appears that Netrins are bifunctional molecules capable of driving attraction through interaction with Frazzled or repulsion through interaction with Unc5.

Midline glia cells also secrete Slit, the ligand for the Roundabout family of transmembrane receptors, Roundabout, Robo2 and Robo3 [4, 10–13]. When Slit function is disrupted, a collapse of the entire neuropile to the midline is observed [10, 11]. When *roundabout* is mutated, an excess of fibers crossing the midline is observed [4]. This suggests that the activation of Roundabout by Slit triggers repulsion of neuronal processes away from the midline. Although both Unc5/Netrins and Roundabout/slit drive repulsion by the midline, it seems that they operate independently from each other [9].

A study using mammalian cell culture suggests that the repulsion driven by roundabout signaling is due to an association of activated Roundabout with N-cadherin, a mediator of homophilic cell adhesion. This association disrupts the link between N-cadherin and the actin cyto-

skeleton, disabling cadherin-mediated adhesion [14]. This suggests that a growth cone expressing Roundabout at its surface and in the vicinity of the midline has its Roundabout receptors activated, which leads to disruption of adhesion. Thus, the response to attraction/repulsion signaling would be by sticking/sliding.

Analysis of the expression pattern suggests that most if not all neurons express *roundabout* [4], suggesting a regulatory mechanism that allows some neurons to send their processes across the midline. Immunodetection of Roundabout showed that the protein is nearly excluded from the commissures [15]. *Commissureless*, whose mutation makes the neuronal processes unable to cross the midline, is cell-autonomously responsible for the downregulation of Roundabout in commissural axons [16]. Commissureless downregulates Roundabout by diverting it to the endosome pathway, where it is thought to be degraded [16], hindering its activation by Slit. Commissureless is itself regulated by an ubiquitin ligase DNedd4 and an interaction between Commissureless and DNedd4 is required for downregulation of Roundabout [17]. The expression of Commissureless is believed to be restricted to the neurons sending their axon across the midline [16, 18]. It also appears to be highly dynamic [16], suggesting that the downregulation of Roundabout has to occur with a precise timing during the course of axon guidance.

With its stereotypical organization, the ventral nerve cord of *Drosophila* allows identified single-cell analysis of various mutants. These analyses and single-cell genetic manipulations can be achieved with the use of the Gal4/UAS system [19]. Gal4 drives transgene expression that can be restricted to a subset of cells per hemisegment. Alternatively, fluorescent dyes can be applied directly to the cell of interest, allowing the visualization of the morphology of an entire identified neuron [3, 20, 21]. Such analyses have shown that axon and dendrites of an unidentified neuron can respond differentially to mutations of *roundabout* or *frazzled*. Among the motoneurons studied, the aCC motoneuron (anterior Corner Cell) possesses an axon that remains ipsilateral and two populations of dendrites, one that crosses the midline and the other one that remains ipsilateral. In *frazzled* mutant embryos, aCC's axonal projection remains unaffected whereas the crossing dendrites are missing, suggesting a requirement of Frazzled for dendritic crossing. In *roundabout* mutant embryos, both axonal and dendritic projections remain as described for wild-type. The RP3 motoneuron, also studied at the single-cell level, projects its axon across the midline to innervate contralateral muscles. RP3 possesses two populations of dendrites, growing away on each side of the midline. In *frazzled* mutant embryos, RP3's axon fails to cross the midline, whereas in *roundabout* mutant embryos, dendrites fail to escape the midline, while the axon remains unaffected and targets its contralateral muscles as in wild-type [22, 23]. These data, as well as the genetic manipulations resupplying the wild-type product of the mutant gene to a small subset of cells show that one process can be cell-autonomously, or more precisely process-autonomously, attracted towards the midline, whereas the other is diverted from it [22, 23]. Such data show that in addition to cell-type-specific regulation of midline signaling through the regulation of Roundabout function, neurons are subcellularly capable of integrating differentially opposite signals in axon and dendrites. The ability to visualize and genetically manipulate a single identified cell makes *Drosophila* an ideal in vivo model to dissect the subcellular regulations involved in the response to midline signaling.

Studies in different vertebrate models have suggested a model by which cells that express both *frazzled* and *roundabout* [4, 7] are able to drive their processes towards or away from the midline. In this model, the activation of Roundabout by Slit unlashes the binding of its cytoplasmic domain with the one of DCC/Frazzled, thus silencing the attraction signals [24]. Single-cell analyses have shown that processes originating from same cell respond to both Roundabout and Frazzled signaling independently [23]. This suggests that the silencing of the attraction signals by Roundabout and the downregulation of repulsion signaling by Commissureless are strictly restricted in terms of localization and timing, conferring processes such independence of response.

CNS Fascicles

In addition to facing the question of whether to remain ipsilateral or to cross the midline, each embryonic neuron selects and fasciculates with one of some 20 longitudinal fascicles that are positioned on either side of the midline. Specific markers expressed on these different fascicles characterize them, as exemplified by the use of anti-Fasciclin antibody (FasII). FasII antibody labels three major parallel pathways on each side of the midline [25]. Each of these three pathways is composed of several fascicles with similar mediolateral positioning but different dorsoventral positions [25–27]. This pattern of expression suggested that cell adhesion molecules are essential for the selection of a longitudinal pathway. How do neurons differentiate between several similar pathways? The characterization of Slit receptors, Roundabout, Robo2 and Robo3, gives insights as to how similar lateral pathways are dis-

criminated [12, 13]. *Roundabout* is expressed in the whole CNS, whereas *Robo3* expression is limited to the medial and lateral regions, and *Robo2* expression is restricted to the most lateral part of the CNS [12, 13, 28]. In addition, all three Robo receptors are absent from the commissures, due to their downregulation by Commissureless [12, 28, 29]. By their combinatorial patterns of expression, these Robo receptors define three regions on each side of the midline, with Robo expression defining the most medial region, combination of Robo and Robo3 defining the intermediate region, and the addition of all three Robos defining the most lateral region of the neuropile [12, 13, 28].

Analyses of loss of function of each of the three Robo receptors, either as a single mutant or as double mutants, as well as overexpression experiments, have shown the following. Roundabout is required primarily to control the crossing of the midline and to confine the longitudinal pathways on each side of the midline [15]. Robo2, on the other hand, contributes to the reading of the repulsion signal, showing that Robo and Robo2 share some function in control of midline crossing [12, 28, 29]. Robo2, however, also possesses its own function in determining which axons will join the most lateral pathways, whereas Robo3 controls the formation of the medial neuropile [12, 28, 29]. These distinct functions are thought to be mediated by both the differences in the protein sequences and the spatiotemporal patterns of expression. Thus, it is proposed that the three Robo receptors establish a Robo code that addresses each axon in one of these regions where local cues and specific adhesion molecules determine the choice of a longitudinal pathway.

Outside the CNS, the neurons in the PNS of *Drosophila* embryos and larvae also offer a wonderful model. Each of the PNS sensory neurons have been identified and their stereotypical axonal projections have been characterized [27, 30]. The position of sensory neurons' terminal projections can be mapped in respect to uniquely identified FasII fascicles [25, 26]. *Roundabout* is found expressed in chordotonal neurons, which project their terminal arbor to an intermediate fascicle, and in multidendritic (md) neurons, which project onto the medial fascicle. *Robo3* is detected only in chordotonal neurons, whereas *Robo2* is not expressed in these types of sensory neurons [27]. Cells depleted of *Roundabout*, either by loss of function or downregulation through overexpression of *Commissureless*, send their terminal projections across the midline and form bilateral projections at the appropriate mediolateral and dorsoventral location, suggesting a similar role of *Roundabout* in confining projections on the ipsilateral side of the midline for PNS sensory neurons as described for CNS neurons [4, 15, 27]. *Robo3*, whose specific expression in chordotonal neurons is directed by Atonal, is required cell-autonomously for the positioning of chordotonal terminal arbors to the intermediate fascicle, as shown by cell-specific rescue experiments [27]. Misexpression of *Robo3* in the dorsal bipolar dendritic (dbd) neuron, one of the md neurons, is sufficient to shift dbd terminal arbor from the medial to the lateral fascicle, showing that Robo3 is necessary and sufficient to control targeting to the intermediate fascicle [27]. As noted previously for CNS neurons, the dorsoventral positioning is not affected by manipulations of *Roundabout* or *Robo3*, suggesting its control by another set of signaling molecules [12, 27, 28]. The dorsoventral positioning might be directed by local cues, once the Robo code addresses CNS axons or PNS sensory terminals to its appropriate position along the mediolateral axis. Again, Slit appears to be the ligand of *Roundabout* and *Robo3* for their function in the positioning of PNS terminal arbors. In addition, Slit also appears to function in the branching of terminals, consistent with the Slit role in dendritic branching observed in vertebrates [27, 31].

Branching

Besides correct pathfinding, the generation of a functional neuronetwork supposes that each constituent of the network will develop the correct shape. Having the right shape, or the right projections, is essential to contacting the correct partners. More importantly, recent studies have shown that the branching pattern as well as the protein composition of both axonal and dendritic arbors play a fundamental role in the electrical properties of a neuron as well as its ability to compute and store information [32, 33]. Thus, identifying the mechanisms that control branching is essential to the comprehension of the development of functional network.

The stereotypical organization of the *Drosophila* CNS as well as the fairly invariant pattern of branching that has been observed in several neuronal types illustrate the presence of genetic programming in axon and dendrite branching. Several different neuronal models have been selected to study either axon or dendrite branching, and have allowed the characterization of molecular mechanisms involved in the development of cell arbors.

The models used for the analysis of both axon and dendrite branching are typically analyzed in late developmental stages, either third instar larvae, pupae or adults.

A genetic screen for branching mechanisms requires that the mutation studied or newly generated will not produce pleiotropic phenotypes or death at earlier developmental stages. The development of genetic tools allowing the generation of homozygous mutant clones in an heterozygous animal [34], complemented by the labeling of the mutant clones with Gal4/UAS system [19] and green fluorescent protein via the segregation of the repressor gene Gal80 (MARCM technique [35]), has overcome the problem of analyzing mutants at late developmental stages. The MARCM technique thus allows the generation of homozygous mutant clones in an heterozygous animal, the cells of the mutant clone being the only cells that express green fluorescent protein. The size of the clone can be controlled, allowing the visualization of single-cell or multiple-cell clones.

Axon Branching

The ability of a neuron to innervate several targets in different locations often depends on the formation of axonal branches. Two mechanisms can lead to the formation of such branches. Sister branches can either develop after the first branch extends, as a collateral extending from a preexisting branch, or they can develop simultaneously, through a splitting growth cone, which gives two sister branches [36, 37].

Mushroom bodies (MBs), a brain structure conserved in Arthropods, appear as a paired bilateral structure. Most axons in MBs possess two major processes that project away from each other at a right angle into distinct target fields [38–40]. MBs play an essential role as they are centers for higher-order functions, including olfactory learning and memory [41, 42].

MBs are derived from four neuroblasts in each hemisphere, each of which produces all three major types of neurons. These neurons are distinguished by their morphological characteristics and their birth order during development [43, 44]. These three types of neurons are γ, α′/β′ and α/β neurons, which project into morphologically distinct lobes. Cell bodies of MB neurons, called Kenyon cells, are packed in the dorsal cortex and extend their dendrites anteriorly into the calyx, which receives olfactory information from the antennal lobes. Distally, MB axons fasciculate into a bundle called peduncle, which splits at its most anterior position to form medial (γ, β′ and β) and dorsal (α′, α) lobes [45].

The four MB neuroblasts, born at an early embryonic stage [46], proliferate continuously throughout development [47, 48]. Clonal analysis demonstrated that each single MB neuroblast produces all 3 types of MB neurons, with generation of γ neurons first, then the α′/β′ neurons, and finally α/β neurons [44]. As proliferation continues, the MB axons are organized into layers, with the projections from the youngest neurons in the center and progressively shifted more laterally, as new axons grow into the peduncle [49]. At first, all these neuron types present branching into medial and dorsal lobes, but during metamorphosis, γ lobes are remodeled through selective pruning and retain only their medial lobes [44, 50]. Thus, MB neurons are capable of producing axon branches that target different regions of the brain.

How is axon branching regulated? A screen using the MARCM technique has been conducted and led to the identification of *Dscam* (Down syndrome cell adhesion molecule) as a regulator of axon branching. In *Dscam* mutant MBs, the γ lobes appear normal, whereas the α/β lobes appear much thicker and denser than wild-type MBs. In addition, mutant clones project their bifurcating axons in a single lobe [51]. Further analyses have shown that Dscam is required for axon bifurcation and guidance of both α′/β′ and α/β axons through cell-autonomous and non-cell-autonomous effects [51]. Single cell mutant clone analysis revealed that α′/β′ and α/β neurons lacking *Dscam* present supernumerary branches deriving from repeated bifurcation. These mutant neurons also failed to segregate branches into the two lobes, showing a cell-autonomous requirement of *Dscam* for the control of branch number and the proper segregation of sister branches [51]. Since *Dscam* is expressed in the nervous system throughout development and there is a similar role of Dscam in another brain structure [51], it is possible that Dscam could be used throughout development to control branching. With its 38,016 possible alternative splice forms [52] however, Dscam might possess numerous functions besides regulation of branching, as underlined by mistargeting of *Dscam* mutant olfactory neurons to the antennal lobe glomeruli, which shows a function of Dscam in targeting specificity [53].

The branching of MB axons to form medial and lateral lobes by bifurcation suggests that axon growth, guidance and branching occur more or less simultaneously. Increasing defects in MB axon branching, guidance and growth were observed with the progressive removal of wild-type copies of the three Rac GTPases *Rac1*, *Rac2* and *Mtl*, with axonal branching being the most sensitive to loss of Rac activity [54]. This suggests that, while occurring concomitantly, growth, guidance and branching are distinct events requiring different levels of activation for the Rac proteins. It also appears that these three events are generated through different effector pathways, since CRIB-

domain effectors of the GTPases, like Pak, are proposed to contribute only to guidance and branching, but not to axon growth [54]. These studies on the contribution of Rho GTPases activity shed some light on how a neuron manages to control different events all involving cytoskeleton rearrangements.

Remodeling Axonal Branches

Holometabolous insects like *Drosophila* undergo a complete metamorphosis between larval and adult stages. Three fates await larval neurons during metamorphosis. They can remain unchanged, they can be removed by apoptosis, or they can go through a phase of remodeling by selective pruning of axons and dendrites, allowing the reintegration of these neurons in adult neuronetworks [55, 56]. This removal of specific neuronal processes in a temporarily controlled manner implies the existence of a genetic program for pruning.

In MBs, while α′/β′ and α/β lobes remain unchanged during metamorphosis, γ neurons specifically retract their dorsal and medial branches and re-extend into a single medial lobe [44, 50]. This offers a model for the study of neuronal remodeling. The pruning of the γ lobe starts with the disruption of the microtubule cytoskeleton and continues with the appearance of blebbing in dendrites and then in axons, followed by fragmentation and removal of both processes. This suggests that pruning occurs by local degeneration rather than retraction [50].

The similarities in phenotypes where the γ neurons fail to remodel during metamorphosis are observed in Uba1 (ubiquitin-activating enzyme-1), as well as in Mov34 and Rpn6 (two subunits of the 19S proteasome-regulatory particle required for polyubiquitinated protein degradation) mutant MB clones, suggesting that proteasome-mediated protein degradation after polyubiquitination is essential for axon pruning [50]. Additional phenotypes observed in these mutant clones suggest pleiotropic functions of protein ubiquitination in different phases of neuronal development [50].

Metamorphosis is under the control of the steroid molting hormone 20-hydroxyecdysone. EcR-B1, one of the isoforms of ecdysone receptor, is specifically involved in the remodeling of larval cells [57, 58]. This EcR isoform is expressed in γ neurons and required for their pruning, and absent in the other MB neurons [59]. In these γ neurons, the activation of Baboon (a TGF-β/activin type-I receptor [60]) by dAct, a member of the activin subfamily [61], induces its heterodimerization with either Wishful thinking or Punt (two constitutively active TGF-β/activin type-II receptors [62–64]), which can act redundantly in γ neurons [65]. dSmad2, baboon's downstream transcriptional effector [60, 66], transduces the TGF-β signal to the nucleus where it directly or indirectly controls the expression of EcR-B1 [65]. Thus, the forced expression of EcR-B1 in *baboon* mutant γ neurons specifically restores remodeling during metamorphosis [65]. The ubiquitin proteasome system has no function in the control of EcR-B1 expression in γ neurons [50]. It is not known if γ neuron pruning requires other cell-type-specific proteins or if EcR-B1 expression is sufficient to induce metamorphosis-related remodeling.

The study of axon branching puts emphasis on the role of downstream signaling pathways with the involvement of Rho GTPases. It appears that these molecules act in different steps of neuronal differentiation, including axon guidance. Furthermore, the fact that different levels of activation of Rho GTPases could control these different steps sheds some light on how neurons reuse similar pathways for different purposes. Although, in MBs, branching and growth occur at the same time, in other neuronal systems such a coupling is less evident or there might be several other decision events coupled with growth. In these cases, additional factors might be required for successfully reusing similar signaling pathways. It is thus possible that timing of events, as well as the use of different sets of effectors or the strict control of downstream pathway protein localization and activation permit neurons to use same pathways to different events.

Dendritic Branching

Each abdominal hemisegment of *Drosophila* embryos and larvae contains 44 peripheral neurons that have been mapped into dorsal, lateral and ventral clusters [67]. Their position as well as their targets and their cellular shape (dendritic arbor and cell size) led to the identification of three neuronal types: external sensory (es) neurons, chordotonal (ch) neurons (both with single, unbranched dendrites) and md neurons [68–70]. Of those, the dendritic arborization (da) neurons present the most developed dendritic arbor. They have been further characterized and chosen as a privileged model for dendritic growth and branching.

The growth of da dendrites starts 2 h after the axons of PNS have reached the CNS, with the emergence at an invariant location of a primary dendritic branch which grows toward the dorsal midline [71, 72]. The growth of the dorsal primary branch continues for several hours, before being replaced by the extension of numerous transient lateral branches along the anteroposterior axis. Some of these lateral branches are stabilized before the

growth of tertiary branches. Altogether, these successive, stereotypic steps of dendritic growth form the complex, reproducible pattern of dendritic arbor observed in da neurons [72]. Furthermore, single-cell analysis revealed that da neurons develop four different types of dendritic arborizations (class I to class IV) with increased levels of complexity and the presence of spiked-like structures for the class-III dendrites [73].

This morphological classification of dendritic complexity has been reported to be coincident with the level of expression of the homeodomain transcription factor *cut*. Class-I neurons display no Cut immunoreactivity, while low levels of Cut in class II, medium in class IV and high in class III are observed [74, 75]. Cut is primarily known for its role in the specification of cell identity [71]. However, *cut* expression persists in post-mitotic cells [74, 76]. Single-cell analysis of *cut* function in post-mitotic da neurons shows a crucial role of *cut* in the acquisition of class-specific morphology, and modulation of its expression is sufficient to change the morphology of da dendrites [75]. As for Cut, a putative transcription factor called Hamlet controls both cell fate specification and dendritic morphology [77]. In *hamlet* mutants, es neurons are transformed in md neurons with full md arbor morphology and post-mitotic expression of *ham* in md neurons transform their dendritic arbor but not their md fate [77]. Although these two transcription factors seem to control both neuronal cell fate and dendritic arbor specification, they appear to do so differently. Hamlet appears to be acting as a switch between two types of dendrite morphology, thus promoting, by its expression, single-dendrite morphology and repressing multiple arbors. In contrast, it is the level of Cut, rather than its expression itself, that selects the level of complexity of da neuron arbor. In addition, Hamlet controls a type of dendritic arbor in relation with a cell-type, whereas Cut controls different levels of dendritic complexity within a same cell-type.

Once the type of dendritic morphology has been determined by transcription factors, unidentified downstream programs are activated to comply. Mutation of some of the genes involved causes overextension of the dorsal branch that grows towards the dorsal midline. In *sequoia* mutants, the dorsal branch of md neurons starts its growth earlier and overextends to cross the dorsal midline and intermingles with the contralateral md neurons [72, 78]. *Sequoia* encodes a putative transcription factor that apparently controls cell-fate specification and dendritic morphology independently [78]. The identification of transcription factors with dual roles, controlling both cell-type specification and specific branching pattern, suggests that neurons rely on intrinsic programming to extend their dendritic field.

As for *sequoia*, *flamingo*, a G-coupled transmembrane protein with extracellular cadherin domains [79], controls the size of the dendritic field. When *flamingo* is mutated, overextension of the dorsal branches of md neurons is observed [72, 80, 81]. Flamingo does not control the dendritic branching pattern in a global way; it only limits the extension of the dorsal branches by controlling the timing of dendritic extension [80, 81]. Since Flamingo can work as a homophilic cell-adhesion molecule in vitro, it is suggested that its control of the timing of dendritic growth and of the dendritic field goes through homophilic interactions. The involvement of both *sequoia* and *flamingo* in the control of the size of dendritic field shows that the branching pattern of da neurons is under the control of both extrinsic and intrinsic factors. The requirement of Flamingo to control the size of dendritic field is not limited to PNS sensory neurons, since *flamingo* mutant MBs also present overextended dendrites [82]. How might the transmembrane signal translate into dendritic extension? Loss of function of *tropomyosin II* has been shown to result in overgrown dendritic fields of da neurons [83]. *Tropomyosin II* stabilizes actin filaments and increases their strength [84]. The genetic interaction of *tropomyosin II* with *flamingo* provides a potential link between *flamingo* and the actin cytoskeleton. Thus, Flamingo signaling leads to a stabilization of actin filaments and therefore controls the size of dorsal branches [83]. Laser ablation experiments and the use of specific mutants reducing the number of PNS neurons generated suggests that the dendritic field of homologous neurons are shaped by competition between neighboring cells, since they repel each other at the dorsal midline and invade the field of the ablated homologous neuron [80]. The analysis of overlapping for the four classes of dendritic field revealed that dendritic arbors from neurons of the same class do not overlap, whereas dendritic arbors of different classes do overlap with each other [73]. This non-redundant coverage of the body wall by dendritic fields is referred to as tiling. In *flamingo* mutants, dendrites of md neurons overextend and invade the homologous contralateral dendritic field [80], suggesting a possible role of *flamingo* in inhibiting overlaps in dendritic fields of homologous class.

PNS dendrites are peculiar in the sense that they do not have specific pre-synaptic partners as would CNS neurons integrated in a network. These PNS neurons collect inputs generated by the external environment of the animal, and they do so by spreading over the body wall. It is still unclear to what extent intrinsic programming

ensures how CNS neurons project their dendrites to the right target field and adopt the right conformation before local dialogue between pre- and post-synaptic partners establishes connections.

Targeting

Establishment of functional circuitry requires that each neuron of the network is able to find and connect with the right target among multiple similar targets. In a multilayered CNS, axons travel through layers and stop at the right layer. Then, in their target layer, the axons identify their appropriate post-synaptic partners. Here, we describe two model systems, although one in more depth than the other, that have given insight into the molecular mechanisms underlying neural targeting during development.

Photoreceptors

The eye of *Drosophila* is a good model to study the targeting of a specific layer in a three-dimensional system. It consists of 800 or so repeated simple units, or ommatidia, containing 8 different photoreceptor cells, R1–R8. R cells send their projections to different layers of the optic lobe. The photoreceptors R1–R6 project to the lamina between two layers of glial cells where they contact their post-synaptic partners to form structures called cartridges. The photoreceptors R7 and R8 navigate through the lamina without stopping and project deeper into the optic lobe to 2 different layers of the medulla, R7 synapsing deeper than R8. In the lamina the matching of photoreceptors with cartridges is not one ommatidium/one cartridge, but rather one point in the visual space/one cartridge [85]. The matching of the number of afferents to their targets is under the control of inductive signals from the R cells [86].

During their axon development, the R cells of each ommatidia project their axon to the optic lobe in a sequential manner that mimics their birth order. R8 enters the optic lobe first, while R1–R6 and finally R7 follow along the R8 tract. Having reached the optic lobe the R cell travels along the surface of the brain before turning medially to penetrate the optic lobe to seek its target layer.

To gain insight into this question, the ability to create mitotic mutant clones in a heterozygous animal using FLP/FRP system [34] has been refined to create homozygous mutant clones only in the eye, with high frequency and, furthermore, with elimination of the homozygous wild-type twin clone [87, 88]. Screening for mutations affecting the targeting of the R cells to the lamina or the medulla have allowed identification of several molecules involved in this process. These studies have shown that different molecules control the targeting to the different layers by the different R cells.

For example, the study of *brakeless* mutant clones has revealed its requirement in the targeting of lamina by R1–R6 photoreceptors. In the absence of *brakeless*, R1–R6 axons are mistargeted to the medulla where they stop at the same level as R7 [89, 90]. This mistargeting can be suppressed when brakeless is resupplied to R1–R6 photoreceptor cells, showing its requirement for targeting to the lamina. However, ectopic expression of Brakeless in R7 and R8 is not sufficient to mistarget their axons to the lamina [89, 90]. This suggests that other essential factors, whose activation is independent of *brakeless*, for the targeting of the lamina are expressed in R1–R6 photoreceptors and not in R7 and R8.

While no apparent change in cell-fate specification is noted in *brakeless* mutant clones [89, 90], *runt*, a transcription factor whose expression is restricted to R7 and R8, is found ectopically expressed in R2 and R5 in *brakeless* mutant clones [91]. Whereas ablation of *runt* from R7 and R8 does not produce a targeting defect, possibly due to redundancy with two other *runt*-related genes, its ectopic expression in R2 and R5 reproduces the mistargeting observed in *brakeless* mutants without changes in cell fate [91]. This suggests that *brakeless* controls the targeting of R2 and R5 by repressing the expression of *runt*. Since the other photoreceptors which usually target the lamina are also mistargeted in *brakeless* mutants, Brakeless might control targeting to the lamina by repressing other transcription factors yet to be identified and/or by activating specific transcription programs in these photoreceptors.

Besides the role of *brakeless* in R1–R6 layer targeting, it has been shown that R1–R6 axons lacking ptp69D, a receptor protein tyrosine phosphatase, are mistargeted to the medulla [87, 92]. Whereas ptp69D is cell-autonomously required for the targeting of R1–R6 termini, its misexpression in R7 and R8 does not retarget their axons to the lamina, indicating that ptp69D is required but not sufficient for lamina targeting [87, 92]. By analogy to its function in embryos [93, 94], ptp69D might receive a signal instructing the axons to defasciculate and take a path different from the R8 trajectory. In that case, defects would be expected also for R7 and, indeed, R7 fails to reach its target layer and stalls in the R8 layer [87].

A similar phenotype where R7 axons are apparently unable to extend over the R8 layer to reach their deeper target layer has also been found in mutants for another receptor protein tyrosine phosphatase (RPTP) called LAR [95, 96]. Analysis of the developmental time course of R7s mutant for LAR has shown that they actually pass through the R8 layer to reach their correct, deeper layer before retracting to the R8 layer at later stages. Thus, LAR is not required for targeting R7 axons to their layer, but for maintaining their interactions with post-synaptic partners once they have reached them [95, 96]. Genetic analysis has shown that increasing the amount of ptp69D in LAR mutant R cells does not compensate for the absence of LAR, but that overexpression of LAR in R cells can compensate for the lack of Ptp69D [95]. This, and also the use of chimeras between ptp69D and LAR, suggests that these proteins may be activating the same intracellular pathway to control R7 layer targeting. But LAR is also likely to possess unique and specific extracellular ligands [95]. The function of LAR in layer targeting seems to be restricted to R7, whereas Ptp69D controls targeting of R1–R6 photoreceptors and R7. This suggests a control of layer targeting with a combinatorial code of protein phosphatases, the specificity of R7 targeting being given by LAR and its specific ligand(s), as shown by the possibility to rescue the mistargeting of R7 axons depleted of ptp69D with LAR overexpression.

As with the embryonic CNS midline, the layered targeting of photoreceptors appears to use the same molecular tools to project and target correctly. As for midline crossing and selection of lateral pathways, the targeting of a layer by a neuron relies strongly on interactions with environment through receptors. In the eye system, while attraction and repulsion mechanisms have not been clearly identified, it is clear that neurons use extracellular signals to select their layers. It is not clear whether an attraction/repulsion mechanism is required for layer targeting, or establishing a 'stop or go' signal is sufficient. If attraction/repulsion signaling is translated into a sticking/sliding signaling, it is possible that the layer targeting is a combination of both type of signals.

Once the photoreceptor axons have reached their target layer, they are confronted by the choice of a specific target inside a layer to constitute a precise topographic map. This phase of neuron growth and targeting is almost the final step of networking, just before synaptogenesis. It involves a set of adhesion molecules. Injection of fluorescent dye into single ommatidia allows the visualization of the pattern of synaptogenesis. In wild-type optic lamina, R1–R6 axons enter their target layer as an ordered bundle. R1–R6 axons from each single ommatidium will then defasciculate, contact their target neurons arranged in columns and establish synapses with them, forming cartridges. *N-Cadherin* mutant photoreceptors R1–R6 present a correct targeting of their layer contrary to what is observed for R7 photoreceptors [97]. However, when depleted of N-cadherin, most of the R1–R6 photoreceptor axons do not defasciculate out of the bundle and do not select the correct synaptic partners [97]. Similar disruptions of the topographic map are observed for R8 photoreceptors [97]. This suggests that N-cadherin activity is required for the defasciculation of the axon termini.

Whereas the control of defasciculation seems to be assured by N-cadherin function, *flamingo*, another member of the *N-cadherin* gene family, is thought likely to be involved in the selection of the synaptic partners by R1–R6 and R8 photoreceptors [98, 99]. *Flamingo* mutant R1–R6 axons from each single ommatidium establish contact with different, randomly selected columns, forming cartridges that are under- or over-innervated, breaking the regular spacing of the termini [98]. Similarly, R8 axon termini do not defasciculate regularly, creating overlaps. In addition, some of the R8 axon termini retract to a more superficial layer, presumably due to a failure to stabilize contact with post-synaptic partners [98, 99]. These defects in the spacing of the axons are reminiscent of tiling defects observed in da neurons in the larval PNS. Flamingo being an homophilic cell-adhesion molecule, one possibility is that growth cones expressing Flamingo establish homophilic interactions triggering repulsion, which keeps each axon regularly spaced.

Neuromuscular System

Once the neuronal process has been successfully targeted to its synaptic partners, synaptogenesis occurs. This has been best studied at the level of genetics, development, physiology and plasticity using the neuromuscular junction between motoneurons and skeletal muscles in *Drosophila* embryos and larvae. Numerous reviews have covered the vast amount of knowledge generated by these studies [100–103]. The *Drosophila* neuromuscular junction still remains an essential and invaluable model for studying target recognition, synaptogenesis and synaptic plasticity from which we can learn about the final steps of the development of functional neuronetworks.

Future Directions

The different neuronal models presented in this review highlight the successive steps that a neuron achieves to form a functional neuronetwork. As shown here, the *Dro-*

sophila nervous system provides a series of models for the analyses of neural development, from determination (not covered here) to differentiation.

The studies on guidance, branching and targeting suggest that the differentiation of axons and dendrites is regulated independently. Axon and dendrites from the same cell respond differently, independently to a similar environment, as shown by the guidance of axons and dendrites at the CNS midline. This suggests the existence of a regulatory system that can segregate axons from dendrites. Several non-exclusive hypotheses can be proposed for such regulatory systems. Since dendrites develop later than axon, timing could account for the independence observed between axon and dendrites. Alternatively, specific control of key molecule localization using different targeting sequences or different motors could generate differences between axons and dendrites. Finally neurons could use the targeting of alternative splice forms with different properties to different compartments to generate such asymmetry in response to signaling.

Neurons rely on extrinsic cues provided by their environment for their differentiation and integration into networks. This is illustrated at the CNS midline where neurons, through Robos and Frazzled receptors, receive Slit and Netrins signaling provided by midline glia to grow toward or away from the midline. This interaction with the environment is also found during axon and dendritic branching as well as during targeting and synaptogenesis, as shown by the involvement of several different cell-adhesion molecules in these processes. This suggests that at each time point in their development, neurons are capable of integrating information provided by the environment to their processes, whether they are axons, dendrites, or sister branches, and respond appropriately to these extrinsic cues.

During CNS midline guidance, the downregulation of Roundabout requires Commissureless associated to DNedd4, a ubiquitin ligase, which suggests a role for the polyubiquitined protein degradation system in axon guidance. Later in development, the polyubiquitinated protein degradation system as been involved in axon pruning during metamorphosis. Similarly, Rho family GTPases control growth, guidance, branching and targeting in different systems and in response to different stimuli. Either the use of the ubiquitinated protein degradation system or the use of Rho family GTPases at successive steps of development is capable of triggering different responses. This shows the versatility of several molecules or several mechanisms that are successfully reused. This suggests that neurons possess strong control systems which allow this versatility.

Identification of the molecular mechanisms that control the differentiation of neurons and their integration in a functional neuronetwork suggests that neurons are continually capable of integrating complex information provided by extrinsic cues and their genetic programming, through the use of similar pathways. Neurons are then able to respond appropriately to the vast amount of cues they received. This suggests the existence of multilayered control systems. Thus we are just at the beginning of understanding how exquisitely precise neuronetworks are established and that the molecules and molecular mechanisms already identified represent only the tip of the iceberg.

Conclusion

Invertebrate nervous systems have been perceived as small and relatively simple units. Analyses of the neuronetworks underlying behaviors, as well as the characterization of developmental mechanisms leading to their formation, illuminate the complexity of elaborating and using a functional neuronetwork. *Drosophila* provides a model integrating genetic developmental analyses and complex neuronetworks. It also stresses that constructing a functional neuronetwork, requires communication, adhesion and exchanges.

Thus, all you need is love [104] ... and good genetics! [105].

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Review

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Crustacean Motor Pattern Generator Networks

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Key Words

Stomatogastric system · Cardiac ganglion · Swimmeret · Gill ventilation · Central pattern generation · Rhythmic neural network · Proprioceptive feedback · Endogenous oscillation · Plateau potential · Graded synaptic transmission

Abstract

Crustacean motor pattern-generating networks have played central roles in understanding the cellular and network bases of rhythmic motor patterns for over half a century. We review here the four best investigated of these systems: the stomatogastric, ventilatory, cardiac, and swimmeret systems. Generally applicable observations arising from this work include (1) neurons with active, endogenous cell properties (endogenous bursting, postinhibitory rebound, plateau potentials), (2) nonhierarchical (distributed) network synaptic connectivity patterns characterized by high levels of inter-neuronal connections, (3) nonspiking neurons and graded transmitter release, (4) multiple modulatory inputs, (5) networks that produce multiple patterns and have flexible boundaries, and (6) peripheral properties (proprioceptive feedback loops, low-frequency muscle filtering) playing an important role in motor pattern generation or expression.

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Introduction

Understanding the genesis of rhythmic motor patterns such as walking and swimming has long been a fundamental goal of neuroscience. This interest was heightened by later discoveries that multiple simultaneous rhythms are present in brain activity, and that these rhythms change as a function of arousal and attention [1–9]. Invertebrate preparations have always played a prominent role in these studies because these preparations are often easily maintained in vitro and often have anatomically distributed nervous systems (as opposed to the highly centralized systems found in vertebrates), large neurons that are easily recorded from and repeatedly identifiable in different animals of the same species, and fixed neuron populations and synaptic connections. Indeed, the crayfish and locust were the first preparations in which it was unambiguously demonstrated that central pattern generators (CPGs) – neural networks capable of spontaneously producing rhythmic, patterned neural outputs in the absence of sensory feedback or patterned central input – exist [10, 11]. Subsequent work in a large variety of systems showed that in all cases, CPGs generate the fundamental rhythmicity and phasing of rhythmic motor patterns [12], resolving a 50-year controversy whether such patterns were generated by spontaneous central rhythmicity or via a reflex chain in which the sensory feedback generated by each individual movement in the pattern triggered the pattern's next movement.

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However, as often occurs when dichotomous choices are forced on data, in reality, the relative importance of spontaneous central rhythmicity and movement-induced sensory feedback varies tremendously from motor pattern to motor pattern. In particular, motor patterns such as terrestrial locomotion – in which cycle-by-cycle variation in response to a variable substrate is critical for behavioral competence, and failure to maintain proper phasing of motor pattern movements in even one cycle could be catastrophic – often so depend on sensory feedback that it is very difficult to induce isolated nervous systems to produce any rhythmic output at all. There is consequently a distinct bias in experimental work on the cellular level toward motor patterns that are less dependent on sensory input (e.g. swimming, flying, respiration, heartbeat, gut movements), although even in these cases sensory feedback often dramatically increases CPG cycle frequency and robustness of neuron firing. Evidence of this bias is present in this article by the absence of a section on crustacean walking, for which, although a great deal is known about locomotor reflexes [13–16], the CPG network remains unidentified.

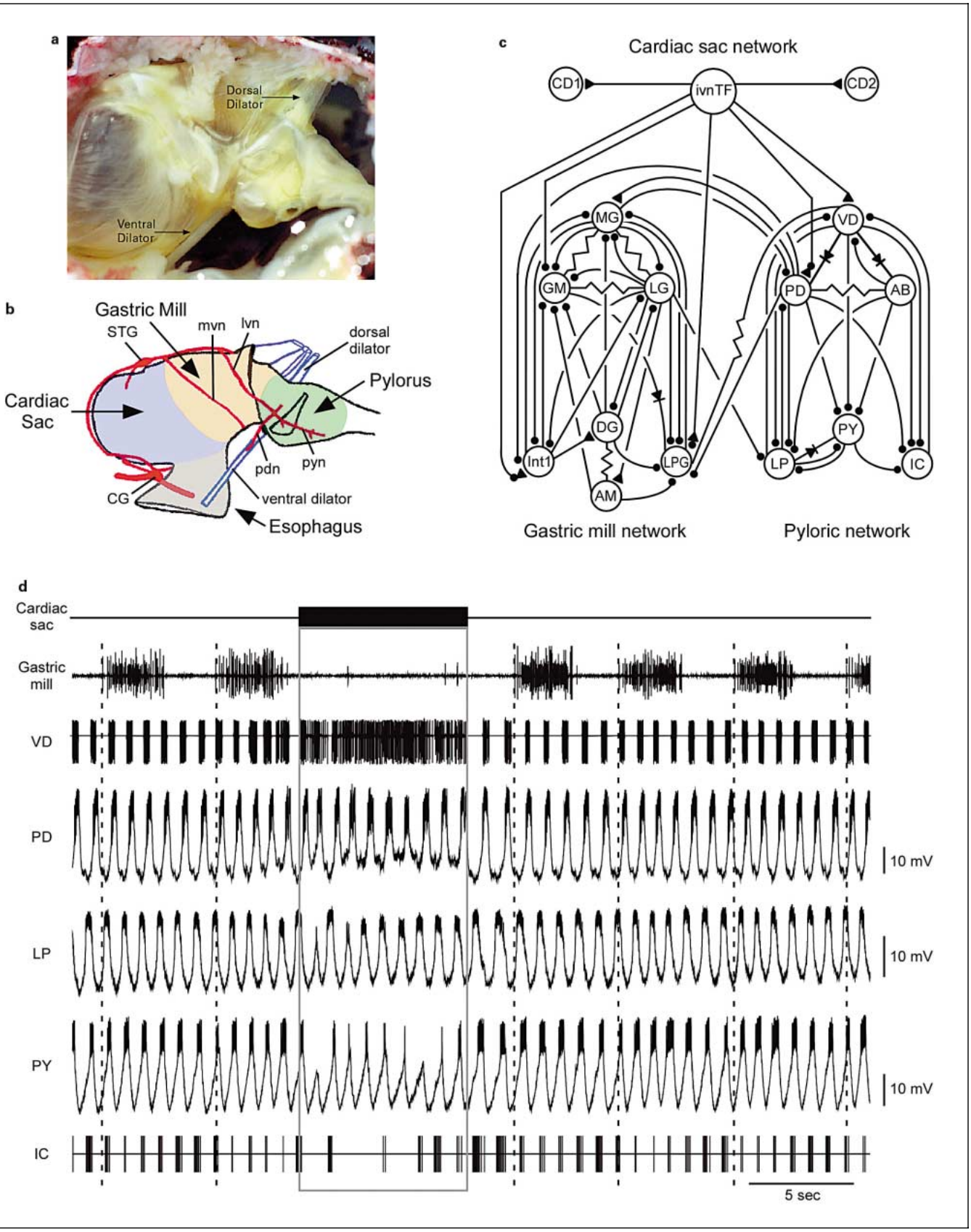
Given that the primary goal of invertebrate work is to gain insight into higher, and particularly human, nervous system function, whether work on a biased subset of invertebrate CPGs can provide generally applicable principles is a valid question. Two observations suggest that this is likely true. The first is evolutionary. Vertebrates (Deuterostomia), worms and mollusks (Lophotrochozoa), and arthropods and nematodes (Ecdysozoa) all have nervous systems that support locomotory, food searching, eating, escape, and reproductive movements, and jellyfish (Radiata) have nervous systems that produce rhythmic locomotory movements. Deuterostomia, Lophotrochozoa, and Ecdysozoa separated at least 500 million years ago. Bilateria (of which Deuterostomia, Lophotrochozoa, and Ecdysozoa are branches) and Radiata separated an unknown but presumably great period earlier. These observations suggest that nervous systems capable of producing rhythmic movements arose very early in animal evolution (presumably present in the last common ancestor of Radiata and Bilateria). Despite their great subsequent divergence, it might be expected that, given evolution's generally conservative nature, remnants of this common ancestry are still present in both invertebrates and vertebrates. Second, and perhaps more convincing, history shows that this approach works. The list of neural and network properties first described in invertebrates and now known to be also present in vertebrates includes the ionic basis of the action potential, many of the known membrane conductances, CPGs, endogenously bursting neurons, plateau properties, nonspiking neurons, nonspiking (graded) transmission, neural network modulation, multifunctional neural networks, and neural networks with changing neuronal complements (e.g. neurons switching between different networks and network fusion).

Crustacean CPG networks have played key roles in many of these discoveries. We review here four crustacean CPGs that are completely or partly known on the cellular level: the extremely well-described stomatogastric system and the ventilatory, heartbeat, and swimmeret CPGs. Particularly important advances from this work include the importance of endogenous, active membrane properties in network neurons; the distributed, nonhierarchical nature of many of the network synaptic connectivity diagrams; the presence of multiple neuromodulatory inputs that alter network output; network flexibility; and the frequent presence of nonspiking neurons and graded synaptic transmission.

The Stomatogastric System

Overview

The stomatogastric neuromuscular system generates the rhythmic movements of the four regions of the crustacean stomach: the esophagus, cardiac sac, gastric mill, and pylorus (fig. 1a, b) [17]. The esophagus moves food from the mouth to the cardiac sac, where it is mixed with digestive fluids. The softened food is then chewed by internal teeth in the gastric mill, and the pylorus filters the chewed food into three streams, one for absorption, one for further chewing by the gastric mill, and one for excretion. The stomatogastric nervous system lies on the surface of the stomach (red, fig. 1b) and contains almost all the neurons of the esophageal, cardiac sac, gastric mill, and pyloric CPG networks. These networks are composed almost exclusively of motor neurons, which both elicit muscle contraction and fulfill the rhythmogenic and pattern formation roles typically performed in other systems by a premotor interneuronal network. This happenstance has greatly facilitated network description, and in the lobster, *Panulirus interruptus,* the complete neuronal complements and synaptic connectivity diagrams are known for the cardiac sac, gastric mill, and pyloric networks (fig. 1c) [17–30]. Three notable characteristics of these networks are (1) the high degree of neuron interconnectivity in the gastric mill and pyloric networks, (2) the lack of a serial, hierarchical arrangement in the gastric mill and pyloric


a
Dorsal Dilator
Ventral Dilator
b
Gastric Mill
STG
mvn
lvn
dorsal dilator
Pylorus
Cardiac Sac
pdn
pyn
CG
ventral dilator
Esophagus
c
Cardiac sac network
CD1
ivnTF
CD2
MG
VD
GM
LG
PD
AB
DG
PY
Int1
LPG
LP
IC
AM
Gastric mill network
Pyloric network
d
Cardiac sac
Gastric mill
VD
PD
LP
PY
IC
10 mV
10 mV
10 mV
5 sec

networks, and (3) the high degree of internetwork interactions.

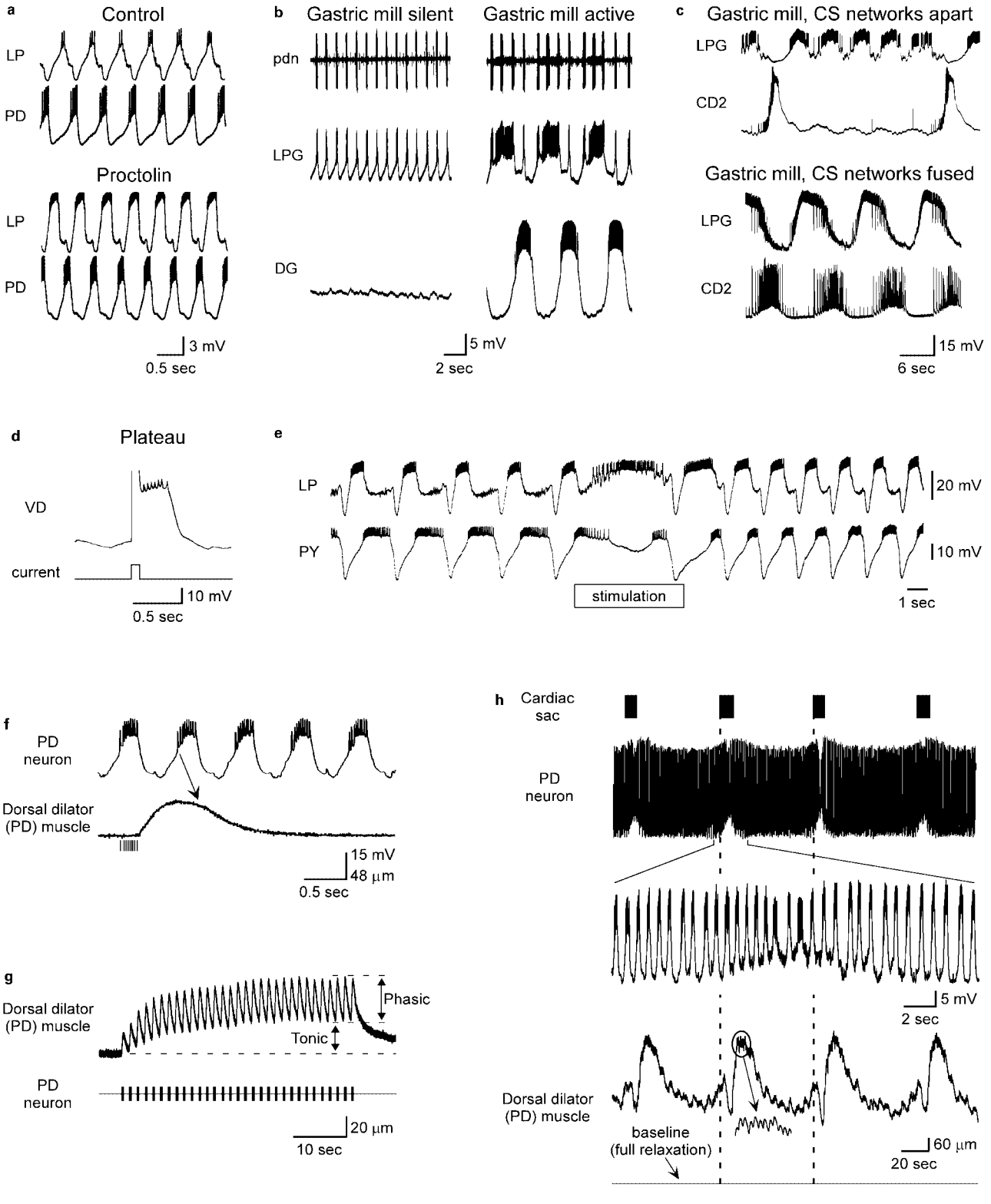
These internetwork connections and the region's anatomical and functional relationships suggest that cardiac sac, gastric mill, and pylorus movements would be coordinated. Simultaneous recordings from the three networks support this expectation (fig. 1d). Cardiac sac neural output consists of long (2–8 s) simultaneous bursts of action potentials in all three cardiac sac network neurons (inferior ventricular nerve through fibers, cardiac dilator 1, and cardiac dilator 2) approximately once per minute (one burst is shown schematically in the top trace; fig. 1d). The gastric mill is a faster (cycle period 5–10 s) multiphasic rhythm, i.e., the gastric mill neurons do not all fire together, but instead each gastric mill cycle consists of a sequence in which first some, and then other, and then still other gastric mill neurons fire, after which the sequence repeats. The trace shown is an extracellular recording of one gastric mill neuron type, the Gastric Mill (GM) neurons. The remaining traces show the activity of all five pyloric motor neuron types: Ventricular Dilator (VD), Pyloric Dilator (PD), Lateral Pyloric (LP), Pyloric (PY), and Inferior Cardiac (IC). The pyloric pattern is even faster (cycle period approximately 1 s), and is also multiphasic. Not shown is the less well-defined esophageal rhythm, which has a 5- to 10-second cycle period.

During cardiac sac bursts (fig. 1d: grey rectangle), the gastric mill and pyloric neural outputs are altered: the gastric mill pauses, VD and PD neuron activity increases, and PY and IC neuron activity decreases [27, 31]. Pyloric activity is also altered during each gastric mill cycle (fig. 1d: delineated by the dashed lines): PY neuron activity decreases just before, and IC neuron activity just after, each GM neuron burst [23, 32]. Not visible on this time scale is a decrease in PD neuron activity that occurs during each GM neuron interburst interval. These changes are sufficient that, due to slow integrative properties in the muscles, some pyloric muscles primarily express cardiac sac and gastric mill motor patterns even though no cardiac sac or gastric mill motor neurons innervate the muscles (see below) [33, 34]. In other species, these interactions are sufficient that an integer number of pyloric cycles occurs in each gastric mill cycle [35, 36]. As such, although the cardiac sac, gastric mill, and pyloric networks are distinct (each network's neurons cycle as separate sets with different cycle periods), and are often studied in isolation, the stomatogastric nervous system is a set of centrally interacting networks that produce a coordinated set of stomach motor patterns.

Multifunctional Networks with Flexible Boundaries

In response to the application of neuromodulatory substances or stimulation of central or sensory inputs, the stomatogastric nervous system produces an extraordinarily wide range of neural outputs by (1) individual neural networks producing multiple outputs [37–54], (2) neurons switching from one network to another [55–60], and (3) networks fusing into larger, aggregate networks [61–63]. The ability of individual networks to produce multiple outputs is of two types. First, analogous to fast and slow swimming, the networks can produce the 'same' pattern at different cycle periods (i.e., normalized to cycle period, the delays between different neuron bursts, and burst durations are constant) [64]. Second, single networks can produce different neural patterns in which the relative delays and burst durations change (analogous to changing swimming strokes). In the example shown in figure 2a, proctolin was applied to the pyloric network, which greatly increased LP neuron burst duration. Figure 2b shows an example of a neuron switching between neural networks. In the first panel (gastric mill network silent), the lateral posterior gastric (LPG) neuron fired once every PD neuron burst. However, when the gastric mill network was active, the LPG neuron instead fired in time with the dor-

Fig. 1. a A photograph of the lobster stomach. **b** A schematic showing the 4 regions of the stomach, the stomatogastric nervous system (red), and 2 pyloric muscles, the dorsal and ventral dilators. Nerve abbreviations: pdn = pyloric dilator; pyn = pyloric; lvn = lateral ventricular; mvn = medial ventricular. Ganglion abbreviations: STG = stomatogastric; CG = commissural. **c** Cardiac sac, gastric mill, and pyloric intra- and internetwork synaptic connectivity. Circles, triangles, and circles and triangles: inhibitory, excitatory, and mixed inhibitory and excitatory chemical synapses. Resistors: nonrectifying electrical coupling; diodes: rectifying electrical coupling. ivnTF = Inferior ventricular nerve through fibers; CD1, CD2 = Cardiac Dilator 1 and 2; MG = Medial Gastric; LG = Lateral Gastric; Int1 = Interneuron 1; AM = Anterior Median. Modified from Thuma et al. [34]. **d** Cardiac sac, gastric mill, and pyloric network activity. Top trace: schematic of cardiac sac burst. The cardiac dilator (CD) 1 and CD2 neurons and the inferior ventricular nerve through fibers simultaneously fire a many second burst (solid bar) approximately every minute. Second trace: extracellular recording of GM neuron activity. Gastric mill bursts last for 2–3 s and gastric mill cycle period is approximately 5 s. The gastric mill network neurons do not fire simultaneously, but instead with fixed phase relationships (e.g. the DG and AM neurons fire out of phase with the GM neurons, not shown). Traces 3–7: extracellular (VD, IC) and intracellular (PD, LP, PY) recordings of pyloric network neurons. Pyloric cycle period is approximately 1 s, and the pyloric neurons fire in a fixed order, PD first, then LP and IC, and then VD and PY. Note the strong alterations of gastric mill and pyloric activity during cardiac sac network bursts, and of pyloric activity during the gastric mill cycle.


a
Control
LP
PD
Proctolin
LP
PD
3 mV
0.5 sec
b
Gastric mill silent
Gastric mill active
pdn
LPG
DG
5 mV
2 sec
c
Gastric mill, CS networks apart
LPG
CD2
Gastric mill, CS networks fused
LPG
CD2
15 mV
6 sec
d
Plateau
VD
current
10 mV
0.5 sec
e
LP
PY
20 mV
10 mV
stimulation
1 sec
f
PD neuron
Dorsal dilator (PD) muscle
15 mV
48 μm
0.5 sec
g
Dorsal dilator (PD) muscle
Phasic
Tonic
PD neuron
20 μm
10 sec
h
Cardiac sac
PD neuron
5 mV
2 sec
Dorsal dilator (PD) muscle
baseline (full relaxation)
60 μm
20 sec

sal gastric (DG) neuron (and hence also the rest of the gastric mill network). Figure 2c shows an example of gastric mill and cardiac sac network fusion – in the top panel, the two networks are cycling independently, whereas in the bottom panel, the neurons of both networks cycle together in a new, conjoint pattern. Note that the cycle period of the new pattern is different from that of either the gastric mill or cardiac sac networks when apart, and thus this is not an example of the neurons of one network joining another.

Mechanisms Supporting Motor Pattern Generation and Pattern Flexibility

The mechanisms underlying network rhythmicity and neuron phase relationships are best understood in the pyloric network. All network synapses are inhibitory. Although the neurons fire bursts of spikes that travel to their extraganglionic targets, intranetwork synapses are graded and correctly timed network oscillations continue even in tetrodotoxin (TTX) [65–69]. When the network is receiving modulatory input from higher centers, its rhythmicity is primarily due to endogenous bursting ability in the Anterior Burster (AB) neuron (although under these conditions, all pyloric neurons are endogenous bursters [70], the AB neuron cycles most rapidly and entrains the other neurons to its period). The AB and PD neurons are electrically coupled and therefore burst together. These neurons inhibit all other pyloric neurons. After the AB/PD neuron burst ends, these other neurons fire because of two endogenous characteristics, plateau properties [71, 72] and postinhibitory rebound. Neurons that plateau have, in addition to a stable hyperpolarized rest membrane potential, a semistable suprathreshold depolarized membrane potential. The neurons can be triggered to move from the rest to the depolarized plateau by brief depolarizations (fig. 2d). Postinhibitory rebound is a property in which inhibition below rest activates hyperpolarization-activated depolarizing conductances, and thus after inhibition, the neuron depolarizes above rest. As a result of these two characteristics, the neurons inhibited by the AB/PD neuron ensemble depolarize above plateau threshold after the AB/PD burst, and thus themselves fire a burst. The phase relationships among these 'follower' neurons (the LP and IC neurons fire first, and then the PY and VD neurons) result from their differing cellular properties (the PY neurons rebound more slowly than the LP and IC neurons [73]), and the synaptic connectivity of the network (the LP and IC neurons inhibit the PY and VD neurons).

Although this explanation is adequate for some network conditions, it is not complete. For example, the network continues to produce a rapid rhythmic output even if the AB neuron is killed. Network rhythmicity in this case is unlikely to be due to the endogenous bursting abilities of the other neurons, as their inherent cycle periods are considerably longer (several seconds) than the 1-second cycle period of the network. In this situation, a different method of rhythmogenesis, half-center oscillation [74], is likely responsible. Key to this mechanism is mutual inhibition between neurons, e.g. the LP and PD neurons. For neurons with postinhibitory rebound and plateau properties, this synaptic arrangement can induce rhythmogenesis as follows. If the PD neuron is induced to plateau, it will inhibit the LP neuron. After the PD neuron plateau ends, the LP neuron will plateau and fire due to postinhibitory rebound. This inhibits the PD neuron, which therefore rebounds and fires after the LP neuron burst ends, and the cycle continues.

Fig. 2. a Stomatogastric (STG) network modulation. Proctolin induces the LP neuron to fire longer, higher-frequency bursts of spikes. Modified from Marder et al. [194]. **b** Neuron switching. When the gastric mill is silent, the LPG neuron fires with the pyloric pattern [pyloric dilator (pdn) extracellular trace]. When the gastric mill is active (note DG neuron cycling), the LPG neuron fires with the gastric mill pattern. Modified from Weimann et al. [59]. **c** Network fusion. In control saline, the gastric mill (LPG neuron) and cardiac sac [cardiac dilator (CD) 2 neuron] networks cycle independently. The modulatory peptide red pigment concentrating hormone induces the networks to fuse. Modified from Dickinson et al. [61]. CS = Cardiac sac. **d** Injecting a brief depolarizing current pulse triggers a plateau in the VD neuron. Modified from Hooper and Moulins [56]. **e** Stimulation (rectangle) of a sensory receptor activated by gastric mill movement disrupts pyloric cycling during the stimulation, and induces a long-lasting increase in pyloric cycle frequency after it. Modified from Katz and Harris-Warrick [46]. **f–h** Slow muscle properties allow muscles to express the motor patterns of noninnervating neurons. **f** The dorsal dilator muscle was isolated from its innervating PD neuron by cutting the motor nerve, and the muscle induced to contract by stimulating the motor nerve (vertical bars under muscle trace) in a pattern identical to the spikes in the first PD neuron burst (top trace). If the innervation of the muscle had been intact, the next PD neuron burst (arrow) would have reached the muscle before it had fully relaxed. **g** When the motor nerve is stimulated with rhythmic shock bursts (rectangles, bottom trace) mimicking motor neuron input, the contractions temporally summate. **h** When driven by real PD neuron bursts (top trace; second trace shows time expansion during cardiac sac network burst), the alterations in PD neuron firing during cardiac sac bursts (see also trace 4, fig. 1d) cause the tonic contraction component of the muscle to vary in cardiac sac time. The primary rhythmic output of the muscle is therefore cardiac sac timed; the inset shows the very small contractions induced by the neuron (PD) that actually innervates the muscle. All panels modified from Morris et al. [33].

In the animal, the AB neuron is presumably never dead, and these multiple rhythmogenic mechanisms therefore presumably do not exist as a fail-safe redundancy to maintain pyloric activity in the unlikely event of AB neuron ablation. They instead presumably reflect, as also does what a priori appears to be the 'overly' complex synaptic connectivity of the network, the ability of the network to produce multiple patterns, and of its neurons to move between networks. Investigation of the mechanisms underlying these properties supports this contention. First, the changes in network activity induced by modulator application often cannot be explained solely by the changes the modulator induces in the neurons it directly affects. Instead, due to the dense synaptic connectivity of the network, changes in directly affected neurons alter the activity of nondirectly affected neurons, and the response of directly affected neurons is altered by their interactions with nondirectly affected neurons [44]. The response of the network is thus distributed across the network, and cannot be understood except by considering the network as a whole.

Second, this distributed action is also seen in network switches. For instance, cardiac sac network activation by a stomatogastric sensory input switches the VD neuron to the cardiac sac network (because the neuron loses its plateau properties, and so fires when the inferior ventricular nerve through fibers of the cardiac sac network fire – see figure 1c – but not after each AB/PD neuron inhibition). Input stimulation also changes IC neuron activity, but these changes occur solely due to the absence of VD neuron input to the IC neuron [56]. Third, examining the effects of VD or LP neuron removal from the network shows that in control saline, most of the synapses these neurons make have inconsistent or no effects on the firing of other pyloric neurons [75]. These synapses presumably did not evolve for no reason, and an attractive hypothesis is that they help generate network activity under other modulatory conditions. Fourth, modulatory inputs to stomatogastric networks can receive presynaptic inhibition from neurons of the network the inputs modulate [76–79]. As a consequence, although the inputs may fire very long bursts or even tonically, their input to the network will occur with the cycle period of the network (because the long bursts are inhibited at the input synapses by the presynaptic inhibition). As such, the synaptic connectivity and cellular properties of the stomatogastric networks, their interconnections, and their inputs cannot be understood except in the context of these being multifunctional networks with flexible boundaries.

Another extremely important point about how the pyloric network functions is its history dependence. History dependence arises in this network via two mechanisms. The first is that some cellular properties, notably the time it takes for neurons to reach plateau after inhibition, vary as a function of the cycle period and duration of the inhibitions the neurons receive. This has been best investigated with the PY neurons, in which rebound time increases with cycle period [80]. The delays between neuron bursts must increase if the 'same' pattern is to be produced as network cycle period changes, and this property thus presumably partially underlies the ability of the pyloric network to produce the 'same' pattern at different cycle periods. This shifting of rebound delay with cycle period, however, is only half that necessary to explain the observed data. The rest of this ability likely stems from a history dependence of synaptic strength on inhibition, cycle period and duration [81–83]. Both of these history-dependent processes vary slowly (over several seconds), and thus network activity at any time is a function of an average of network activity for several prior cycles. Activity in the pyloric network (and presumably the other stomatogastric networks) is thus not only distributed across the component neurons and synapses of the network, but also across time.

Peripheral Integration via Sensory Feedback

The lobster stomach is richly endowed with sensory neurons [84], but in most cases, their function is unknown. Two systems that have been investigated function, in part, to allow movements in one stomach region to alter the movements of other regions. These data thus support the impression made by the extensive internetwork synaptic connections, and coordinated changes in network activity, that coordinated stomach movements are functionally important. The first sensory pathway is triggered by pyloric distention, but induces cardiac sac network bursts [85], which result in cardiac sac dilation. An attractive hypothesis is that this pathway serves to transfer food from the pylorus to the gastric mill and cardiac sac if the pylorus becomes excessively full. The second is triggered by gastric mill movements [46, 86, 87]. One target of this input is the gastric mill network itself, for which it presumably serves as a typical, cycle-by-cycle proprioceptive feedback loop. However, this input also targets the pyloric network. Due to the long duration movements of the gastric mill, this receptor fires long bursts lasting for several pyloric cycles, during which the pyloric cycling is disrupted (fig. 2e). Subsequent to the bursts, pyloric cycle frequency increases for up to a min-

ute. The input thus serves to both coordinate the two networks and modulate pyloric activity.

Peripheral Integration via Slow Muscle Properties

Many stomatogastric muscles contract and relax very slowly – some taking many seconds to fully relax. For the slow cardiac sac and gastric mill networks, this is not a concern, as even very slow muscles could still fully relax between one burst and the next of their innervating neuron. The muscles innervated by the rapid pyloric network, however, cannot fully relax between neuron bursts (fig. 2f), and their contractions therefore temporally summate (fig. 2g). If the pyloric network were not slowly modified in time with the gastric mill and cardiac sac rhythms, pyloric muscle contractions would thus, once the temporal summation was finished, consist of phasic contractions in time with the pyloric bursts riding on a sustained, tonic contraction (fig. 2g) [88]. However, pyloric activity is modified in gastric mill and cardiac sac time, and the tonic contraction component of the slow muscles varies with these modifications [33, 34]. These variations can be the primary rhythmic motor output of some slow muscles (fig. 2h), even though no gastric mill or cardiac sac motor neuron innervates them.

Gill Ventilation

Overview

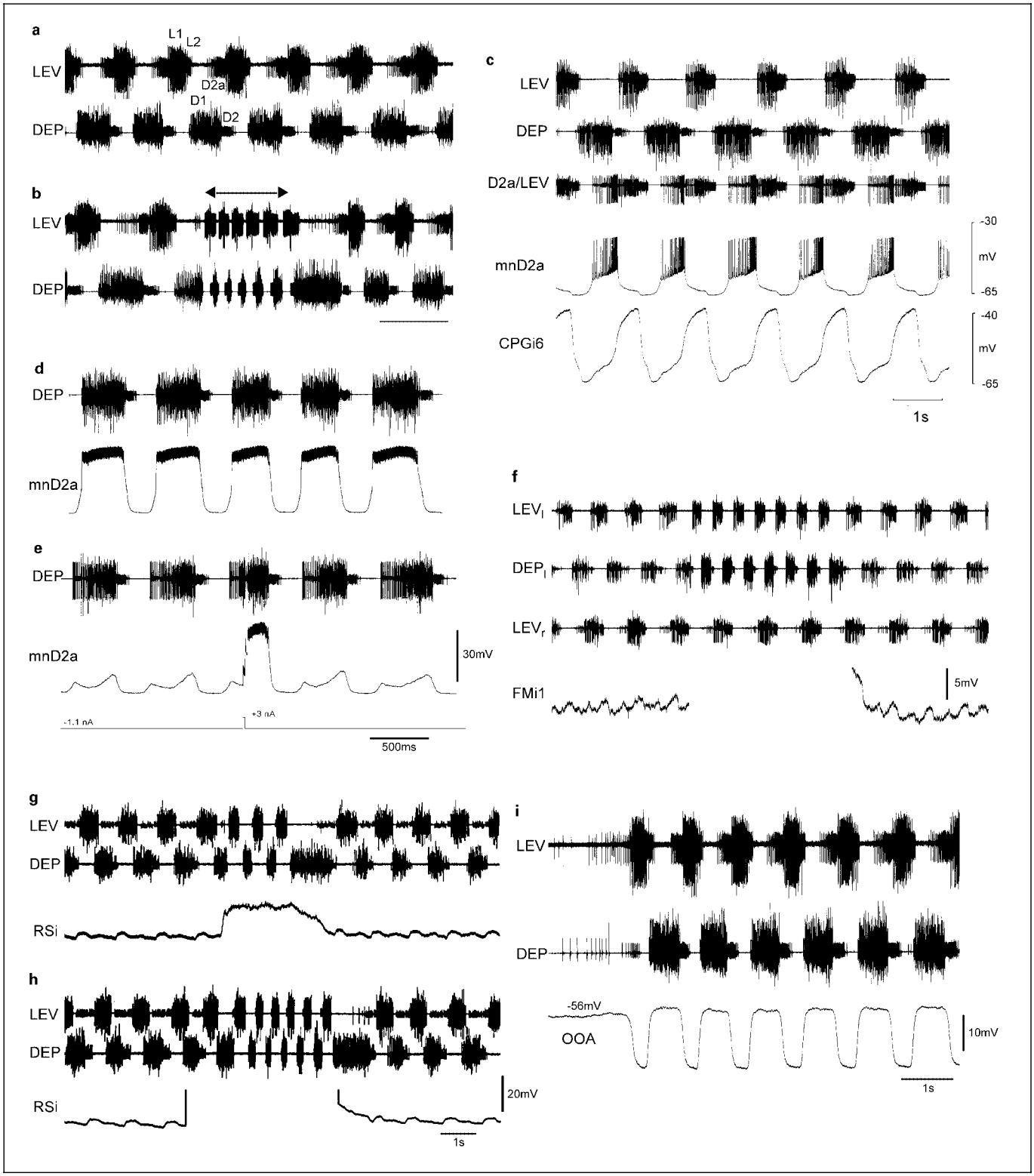
Ventilation in decapod crustacea is produced by rhythmic dorsoventral movements of the scaphognathite (SG) of the second maxilla, which pumps water through the branchial chamber and over the gills. Five depressor and levator muscles control SG movement [89]. SG movement can pump water in either of two directions, corresponding to forward and reverse ventilation. In forward ventilation, water enters at the base of the legs and exits via anterior exhalent channels under the antennae. Forward pumping is the prevalent mode in *Carcinus maenas.* In reverse ventilation, the recruitment sequence of levator and depressor muscle subgroups is changed such that water enters through the anterior channels and exits at the base of the legs. The forward to reverse transition always occurs between the depressor and levator bursts, at which time there is nearly equal pressure between the pumping chamber and the branchial chamber [90]. Motor program switching at this time would thus minimize backwash of fluid into the pumping chamber, which could mechanically perturb the SG blade.

The Ventilatory CPG Is Composed of Interneurons and Motor Neurons

When the thoracic ganglion and appropriate nerves are removed from the animal, spontaneous rhythmic motor neuron spike bursts corresponding to forward and reverse ventilation are observed (fig. 3a, b). As in the animal, forward ventilation is most common, with bouts of reverse ventilation, and ventilatory pauses, occurring infrequently. Unlike the stomatogastric system, the CPG contains large numbers of premotor interneurons. Although originally thought to be a single endogenously oscillating nonspiking neuron [91], the interneuronal ventilatory CPG was later shown to consist of at least two [92], and is now known in the crab, *C. maenas,* to consist of 8, nonspiking interneurons [CPG interneurons (CPGi) 1–8] [93]. Figure 3c shows extracellular recordings of the activity of all the ventilatory motor neurons, and intracellular recordings from a depressor motorneuron and one CPGi. The network displays all the defining characteristics of a true CPG. The interneurons exhibit large-amplitude (10–35 mV) membrane potential oscillations during forward and reverse ventilation, and no oscillations during pauses. In the in vitro preparation, no sensory feedback loops are present, and the interneurons do not receive phasic non-CPG descending input. Injecting intracellular current pulses into any interneuron can reset the rhythm.

All the interneurons are restricted to a single hemiganglion, there is one (and only one) of each interneuron type per hemiganglion, and intracellular current injection affects only the activity of the hemiganglion containing the interneuron. These data indicate that separate CPGs produce the left and right SG ventilatory patterns [93], and are consistent with data showing that the left and right ventilatory rhythms are generated and controlled independently [90, 91, 94]. Moreover, it reinforces the hypothesis that the loose phase coupling observed between bilateral CPGs [94] is mediated by the nonspiking frequency modulating interneurons (FMis; see below) [95] rather than midline crossing CPGi interconnections.

The interneuron synaptic connectivity pattern is unknown because the primary method of demonstrating synapses, one-for-one constant-delay matching of postsynaptic potential and presynaptic spikes, is unavailable in a network of nonspiking neurons. However, neuron membrane potential trajectories, data from current injection experiments (to see if neuron hyperpolarizations and depolarizations reverse), and voltage clamp recordings showing appropriately timed inward and outward currents suggest that the interneurons receive both excitatory and inhibitory inputs from the rest of the network.


a
L1
L2
LEV
D2a
D1
D2
DEP
b
LEV
DEP
c
LEV
DEP
D2a/LEV
mnD2a
-30
mV
-65
CPGi6
-40
mV
-65
1s
d
DEP
mnD2a
e
DEP
mnD2a
30mV
-1.1 nA
+3 nA
500ms
f
LEV$_l$
DEP$_l$
LEV$_r$
FMi1
5mV
g
LEV
DEP
RSi
h
LEV
DEP
RSi
20mV
1s
i
LEV
DEP
-56mV
OOA
10mV
1s

Whether network rhythmicity arises from endogenous oscillatory properties or a nonspiking equivalent of half-center oscillation is also unknown. Current injection into single interneurons does not reliably elicit excitatory or inhibitory responses from a single or groups of motor neurons, suggesting that motor neurons receive input from multiple CPGis. Sustained CPGi hyperpolarization can, however, excite some ventilatory motor neurons, which may indicate that, as has been demonstrated in other arthropod nervous systems [67, 96], the CPGis continuously release transmitter in a graded manner. Current pulse injection into the motor neurons also resets the rhythm, and thus these neurons are also part of the ventilatory CPG, but the synaptic connectivity among them, and to the interneurons, is again unknown.

Motor Neuron Properties

Ventilatory motor neurons exhibit large-amplitude (15–30 mV) membrane potential oscillations (fig. 3c, trace 4; fig. 3d, trace 2). Motor neuron bursting had initially been attributed to cyclic inhibition from the ventilatory CPG onto neurons that otherwise would fire tonically [97, 98]. However, a subsequent study showed that the motor neurons possess plateau properties [99]. When ventilatory motor neurons are hyperpolarized by intracellular current injection, the large-amplitude membrane potential oscillations are abolished, and only small (5–8 mV) oscillations in phase with the motor pattern remain. Injecting a brief depolarizing current pulse induces a large amplitude plateau potential and a burst of action potentials that lasts until it is terminated by inhibitory input from the rest of the network (fig. 3e, bottom trace). Injecting brief hyperpolarizing current pulses during the bursts of normally cycling motor neurons, however, does not terminate the plateaus as it should if only the plateau supports the bursts. It is thus likely the motor neurons also receive excitatory synaptic drive during their bursts. Plateau potentials are found in all motor neurons of both the forward and reverse populations.

Ventilatory motor neuron burst endings are therefore likely due to cyclic synaptic inhibition from the ventilatory CPG, as has been proposed previously [97], whereas burst beginnings are due to plateau potentials triggered either by postinhibitory rebound or excitatory input from the rest of the network. However, the presence of a plateau potential removes the need for the CPG to supply excitatory drive to the motor neurons throughout the burst duration.

Starting, Stopping, and Changing the Frequency of Ventilatory Rhythms

Descending fibers that stop, start, and alter ventilation period are present in nerves connecting the brain and the thoracic ganglia [94]. Three FMis (FMi1–3) have been identified that have somata and processes in the subesophageal ganglion and are apparently intercalated between the descending fibers and the ventilatory CPG. Changing FMi membrane potential by intracellular current injection can start and stop ventilation, and alter ventilation frequency in a graded manner across the physiologically observed range [95]. All three FMis project bilaterally into the left and right CPG neuropil, but changing FMi1 membrane potential alters only the rhythm of the CPG ipsilateral to its soma (fig. 3f). FMi2 and FMi3, alternatively, modulate the rhythm of both CPGs. Ventilation frequency increases with FMi1 depolarization, and decreases with FMi1 hyperpolarization; the reverse is true of FMi2 and FMi3. The FMis receive cyclic synaptic input in phase with the ventilatory rhythm and thus cycle

Fig. 3. The crustacean ventilatory system. All records from isolated ganglion preparations in *C. maenas*. **a**, **b** Extracellular recordings of ventilatory motor output during forward and reverse ventilation. The SG is driven by levator (L) and depressor (D) motor neurons, each divided into two subgroups, L1 and L2, D1 and D2. **a** During forward ventilation, the muscle recruitment sequence is L1-L2-D1-D2 [89]. **b** In reverse ventilation (arrow), ventilatory cycle frequency increases and the muscle recruitment sequence is D2-D1-L2-L1. **c** Extracellular recording of all ventilatory motor neurons (top 3 traces) and intracellular recordings from a motor neuron innervating muscle D2a (mnD2a) and a nonspiking CPGi, CPGi6. **d**, **e** Plateau potentials in ventilatory motor neurons. **d** Extracellular recording of depressor (D) motor neuron activity and intracellular recording from a D1 motor neuron during forward ventilation. mnD2a = Motor neuron innervating muscle D2a. **e** Injection of a 1.1-nA hyperpolarizing current into the motor neuron decreases its oscillation amplitude to a maximum of 9 mV. Injection of a 20-ms current pulse in addition to the sustained hyperpolarizing current (net current amplitude +3 nA) triggers a plateau potential. This resultant spike burst is terminated at its normal phase by synaptic inhibition from the ventilatory CPG. **f** Extracellular recordings of left (top 2 traces) and right (third trace) ventilation CPGs and intracellular recording from left FMi1. FMi1 depolarization (3 nA) increases ventilation frequency on the left side but not on the right. **g**, **h** Reversal switch interneuron RSi1. **g** RSi1 depolarizes during a spontaneous switch to reverse ventilation and repolarizes on return to forward ventilation. **h** Depolarizing RSi1 (2.5 nA) during forward ventilation initiates reverse ventilation, which persists for the duration of the current step. **i** Ventilatory gating of sensory input. Intracellular recording from an oval organ afferent (OOA). During a ventilatory pause, the membrane potential of the afferent is unchanging. Ventilatory activity is associated with a large amplitude hyperpolarizing oscillation in OOA membrane potential.

with it. This input is of a polarity (excitatory for FMi1, inhibitory for FMi2 and FMi3) that would act as a positive feedback loop on ventilatory cycle frequency. Injecting brief current pulses into any FMi resets the rhythm, and thus these neurons are part of the CPG network.

The changes in CPG neuron or synaptic properties that cause the rhythm to start, stop, and change frequency are little known. An important component of rhythm stopping, however, is cessation of motor neuron plateau potential ability [99]. Plateau property expression thus depends on influences from the ventilatory CPG or from descending inputs to the motor neurons parallel to, or the same as, those that activate the CPG. Modulation of plateau expression has been observed in several other invertebrate and vertebrate motor systems [56, 100–105].

Ventilatory Rhythms Maintain Phase as Their Cycle Frequency Changes

Changing cycle period raises the difficulty of whether the 'same' pattern is produced at different cycle periods. That is, if neuron B fires 2 s after neuron A when the cycle period is 4 s, neuron B begins to fire half way through the pattern. If neuron B continues to fire 2 s after neuron A when the period is 3 s, neuron B now begins to fire three quarters through the pattern (and thus the slow and fast patterns are not the 'same' in that, were a plot of the slow pattern reduced along the time axis by 25%, the two patterns would not overlay). Even more extremely, if the period decreased to 1 s, neuron B could not fire at all. Systems that maintain constant time delays between events in the motor pattern thus produce motor outputs in which phase (delay between events divided by period) varies with cycle frequency, whereas systems that maintain phase must increase or decrease the time between events as cycle frequency changes. Both constant delay and constant phase-maintaining systems are observed [64, 106–112].

Ventilation occurs in vivo at frequencies of 40 to over 300 cycles per minute, an 8-fold range [113, 114]. Cinematography of SG movements is unavailable, and it is thus unclear if motor pattern movements maintain phase (that is, that each movement proportionally changes) as cycle period is altered across this range. However, in vitro recordings show that motor neuron output maintains phase over a 7-fold range of network cycle period [115]. If movement faithfully reflects motor neuron activity in this system, movements would thus be expected to maintain phase. Phase maintenance over this large (300–2,100 ms) period range requires that neural pattern time delays change up to 800 ms. Intracellular recordings show that motor neuron membrane potential trajectory changes little as cycle period is altered. However, recordings from the interneurons reveal that the rise and fall slopes of their membrane potential oscillations change proportionally with cycle period changes [115].

These data imply that phase maintenance is critical to gill bailer function. Comparison of phase-maintaining and nonphase-maintaining motor patterns suggests a possible reason. Nonphase-maintaining motor patterns (e.g. walking) often have distinct power vs. return strokes; phase is not maintained because at all cycle periods the return stroke is about as rapid as it can be, and thus period can only change by changing return stroke duration. Phase-maintaining motor patterns (e.g. airstepping) generally do not have clearly differentiated power and return phases. The SG moves as follows. Beginning with the anterior tip of the blade being levated and the posterior depressed, the anterior tip then depresses and the posterior tip levates, after which the anterior again levates and the posterior depresses [89]. Pumping occurs both when the anterior depresses and the posterior levates, and when the anterior levates and the posterior depresses, and thus the pattern does not have distinct power vs. return strokes [116]. Pump function is well maintained as cycle period changes – ventilation volume and branchial pressure gradient are exactly proportional to ventilation frequency [117], and pump efficiency remains a constant 85% over a wide range of ventilatory frequencies [118].

Without a detailed understanding of SG biomechanics, it is impossible to prove that motor neuron phase maintenance is required for this high maintenance of pump function as cycle period changes. Consideration of SG anatomy and neuromuscular control, however, suggests this may be the case. The SG is flexible, and the anterior and posterior tips are independently controlled. Thus (although it never occurs in the animal), if motor neuron phase relationships were not maintained, the anterior tip, for instance, could depress long before the posterior tip levated, which would destroy pumping. Ventilatory phase maintenance may thus serve two purposes. First, tight maintenance at blade tip transitions causes one tip to start its transition immediately after the opposite tip is maximally levated or depressed. This maximizes expelled fluid and minimizes the time that both tips are in the same (levated or depressed) position. Second, phase maintenance during the remainder of the motor pattern may produce smooth, coordinated blade movements, preventing discontinuities in SG movement that might decrease pump efficiency.

Switching between Forward and Reverse Ventilation

A major change in reverse ventilation is that the motor neurons that innervate the L2 and D2 muscles, and which are active during forward ventilation, stop firing, and the muscles are driven instead by a set of reversal specific motor neurons [89, 119]. These 'reversal' motor neurons are cyclically inhibited during forward ventilation but do not fire; their recruitment in reverse ventilation is apparently due to a tonic depolarizing drive during this time. Reversal motor neuron depolarization by intracellular current during forward ventilation results in these neurons firing bursts at the phase of the ventilatory cycle appropriate for reverse ventilation. A neuron switch also occurs on the CPGi level, the peak-to-peak amplitude of CPGi1 increases, and that of CPGi5 decreases, during reverse ventilation. The decrease in CPGi5 amplitude is sufficient that the neuron likely stops releasing transmitter, and is thus no longer a functional element of the ventilatory CPG. Whether the changes in CPGi1 are also large enough to constitute a switch (with it functioning with the CPG only during reversed ventilation) is not clear, but its input to the CPG is clearly greater during reversed ventilation. The oscillatory amplitudes of the other 6 CPGis are unchanged in forward and reverse ventilation.

A reversal switch interneuron (RSi1) that depolarizes when ventilation reverses, and remains depolarized during reverse ventilation, has been identified (fig. 3g) [120]. RSi1 depolarization by current injection reverses ventilation for the duration of the step (fig. 3h), and RSi1 hyperpolarization during reverse ventilation terminates the reverse motor program. Brief depolarizing current pulses injected into RSi1 reset the forward rhythm, but only if applied at certain phases in the pattern, suggesting that RSi1 has access to the ventilatory CPG during only specific portions of the cycle. Hyperpolarizing pulses never reset forward ventilation, suggesting that RSi1 is not a component of the CPG during forward ventilation. An attractive hypothesis is that RSi1 causes reversal by inhibiting the forward ventilation L2 and D2 motor neurons and CPGi5 and exciting the reversal-specific motor neurons and CPGi1. Anterior branches of RSi1 are close to where the FMis span the thoracic ganglion, and FMi2 is tonically hyperpolarized during reversed ventilation. The posterior branch of RSi1 could provide a mechanism for the correlation between the onset of reverse ventilation and tachycardia [121, 122], as the cardiac accelerator and inhibitor neurons lie posterior to the ventilatory neuropil.

Gating of Sensory Input to the Ventilatory CPG

The only SG proprioceptor is the oval organ, located adjacent to the SG flexion axis [123]. The oval organ is innervated by three afferent neurons with somata in the thoracic ganglion; depending on the species, the afferents can be either spiking (lobster [124]) or nonspiking (crab [125]). Depolarizing or hyperpolarizing current pulses injected into a single afferent reset the ventilatory rhythm. Imposed SG movement in the lobster elicits afferent action potentials during both levation and depression [123], and pressure recordings in intact crab branchial chambers reveal two negative pressure pulses per cycle [116]. Although this would seem to indicate that oval organ afferent input would reach the ventilatory CPG twice per cycle, intracellular recordings from crab oval organs show that afferent central processes are inhibited in phase with the ventilatory motor pattern (fig. 3i). The inhibition blocks afferent input to the ventilatory CPG for approximately 50% of the cycle period, which likely includes one of the two pressure pulses per cycle [89]. This restriction of sensory input to one phase of the motor pattern may prevent sensory input from reaching the CPG at an inappropriate time, and may be analogous to primary afferent depolarization [126], which is also believed to modulate reflex pathways so as to prevent inappropriately timed input during rhythmic motor pattern generation [127–129].

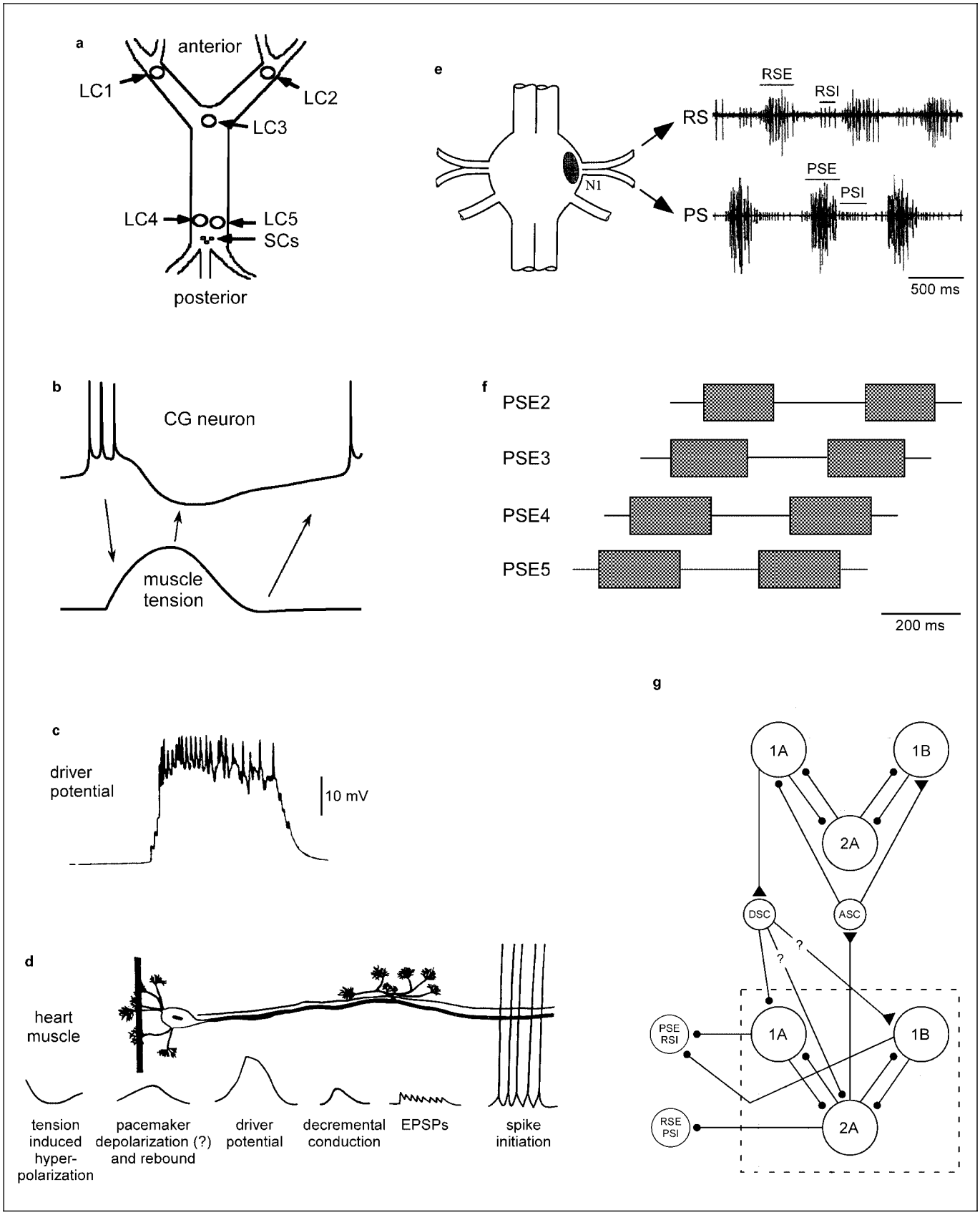
Cardiac CPG

Overview

The cardiac ganglion drives the heart in a very stereotyped and stable rhythm [130]. In most crustacea, the ganglion contains 9 neurons (range, 6–16) located in or on the heart. The neurons are functionally and anatomically subdivided into 4 small posterior interneurons and 5 large anterior heart motor neurons (fig. 4a). The dendrites of both neuron types extend out of the ganglion onto the surface of the heart. The large cells have a peripheral spike-initiating zone, and may have multiple spike-initiating zones. Bursts begin with a high firing rate that decreases during the burst, and have only a small number of spikes. For each neuron, the temporal pattern of individual action potentials in the bursts is highly reproducible across cycles [131].

Network Synaptic Connectivity

Each small cell makes excitatory glutamatergic synapses with all large neurons [132]; both axon-axonal and


a
anterior
LC1
LC2
LC3
LC4
LC5
SCs
posterior
e
RSE
RSI
RS
N1
PSE
PSI
PS
500 ms
b
CG neuron
muscle
tension
f
PSE2
PSE3
PSE4
PSE5
200 ms
c
driver
potential
10 mV
g
1A
1B
2A
DSC
ASC
PSE
RSI
RSE
PSI
d
heart
muscle
tension
induced
hyper-
polarization
pacemaker
depolarization (?)
and rebound
driver
potential
decremental
conduction
EPSPs
spike
initiation

axosomatic synapses are present [133]. All cardiac neurons are also electrically coupled [134, 135]. These synapses significantly attenuate action potentials, but transmit slow potentials (pacemaker and driver potentials) throughout the network. No synaptic potentials have been observed in small cells, but the possibility of graded synaptic input has not been tested. Nitric oxide has been suggested to be an intrinsic neuromodulator of the network [136].

Rhythmogenesis in vivo May Involve a Proprioceptive Feedback Loop

An intact heart that is not mechanically stressed by suspension via ligaments or stretched by internal perfusion pressure does not generate rhythmic movements [137], and cardiac neurons in such quiescent hearts do not fire rhythmic spike bursts [131]. However, in situ preparations where the heart is stretched will generate a rhythm, as do mechanically stimulated intact preparations [131, 137]. These data suggest that proprioceptive feedback (via cardiac neuron dendrites on the heart) from CPG-induced movements help maintain CPG activity. Direct evidence of this is provided by data showing that mechanical stimulation of the heart wall affects the cardiac rhythm on a cycle-by-cycle basis [138]. Intracellular recordings from large cells show that spontaneous heart contractions, or stretch-induced increases in heart muscle tension, hyperpolarize the neurons and induce a rebound burst in the neurons after the stretch (fig. 4b). Depolarization latency decreases with increased stretch amplitude and duration, and repetitive mechanical stimulation entrains the network. The cardiac neurons may thus be part of a one-neuron reflex feedback loop in which the contraction caused by one cardiac burst helps induce the neuron depolarization responsible for the rhythm's next burst.

Contrary to this explanation, however, is the observation that isolated cardiac ganglia are rhythmic. This rhythmicity is due to slowly depolarizing pacemaker currents in the small cells that eventually trigger them to burst (see below). A possible reconciliation of these conflicting data is that dissection of the cardiac ganglion from the heart results in an injury current which is the (nonphysiological) basis of the small cell pacemaker currents. Large cells maintained in cell culture produce spontaneous driver potentials [139], but these are not seen in intact networks, and thus may result from changes in large cell membrane conductances induced by long-term synaptic isolation and cell culture conditions.

Active Neuron Properties Underlie the in vitro Rhythm

Much of this work has been done with isolated cardiac ganglia. Under these conditions, cardiac CPG rhythmicity may arise in part from a nonphysiological 'leak' current in the small cells. Nonetheless, if interpreted in light of most recent results, the isolated ganglion data are valuable for understanding cardiac rhythmogenesis. The in vitro rhythm results from active, plateau-like properties termed driver potentials (fig. 4c). In the in vitro preparation, the small cells spontaneously slowly depolarize (presumably from the injury current noted above), and eventually reach the driver potential threshold, which induces a spike burst. These spikes excite the large cells, which are then driven over the driver potential threshold and produce a spike burst as well. Large-cell synchrony is assured by the electrical coupling among the neurons.

Large-cell driver potentials have been most studied because the potentials in these neurons arise at a site electrically distant from the spike initiation zone, and driver potential currents are thus easily separable from action potential currents. The driver potential threshold and amplitude are proportional to the stimulation rate – increasing the time between depolarizing inputs decreases the activation threshold and increases the potential am-

Fig. 4. Cardiac ganglion and swimmeret systems. **a** Schematic of *C. maenas* cardiac ganglion. Cells numbered using the nomenclature of Alexandrowicz [145]. LC = Large cell; SC = small cell. **b** Schematic of cardiac ganglion (CG) neuron membrane potential and heart tension. Cardiac ganglion motor neuron (large-cell) firing increases heart tension. The increase in tension hyperpolarizes the neuron, which may assist burst termination and increase neuron after-burst hyperpolarization. When the heart relaxes, the neuron undergoes postinhibitory rebound, which may advance the following burst. Modified from Sakurai and Wilkens [138]. **c** Driver potential in a large cell. Modified from Benson and Cooke [195]. **d** Schematic of the generation of pacemaker and driver potentials in the cardiac ganglion. EPSPs = Excitatory postsynaptic potentials. Modified from Benson and Cooke [195]. **e** Left: diagram of an abdominal ganglion showing the first pair of segmental nerves (N1), which provide the sole innervation of the swimmerets in that segment. Shaded region denotes swimmeret neuropil. Each N1 divides into anterior and posterior branches containing return-stroke (RS) and power-stroke (PS) motor axons. Right: extracellular recordings of spontaneous rhythmic bursts in swimmeret motor neurons recorded from RS and PS nerves. PSE = Power-stroke excitor; RSE = return-stroke excitor; PSI = power-stroke inhibitor; RSI = return-stroke inhibitor. Modified from Mulloney [166] and Mulloney et al. [196]. **f** Schematic of metachronal rhythm in a chain of abdominal ganglia. PSE = Power-stroke excitor. **g** The swimmeret CPG circuit. Data from Mulloney [166], Jones et al. [177] and Skinner and Mulloney [197]. DSC = Descending coordinating interneuron; ASC = ascending coordinating interneuron; PSE = power-stroke excitor; RSI = power-stroke inhibitor.

plitude – but depolarization of isolated (ligatured or TTX treated) large cells above –45 mV generally triggers a driver potential. Physiological cardiac cycle periods are approximately 1 s, but lowest activation thresholds and maximum driver potential amplitudes occur with an interstimulation interval of ≥10 s. The driver potentials primarily depend on a voltage-dependent Ca^{2+} current, but three potassium currents (fast I_a, slowly inactivating I_k, and the calcium-dependent I_{KCa}), a noninactivating sodium current, and I_h, a hyperpolarizing activated outward current, are also present in cardiac ganglion neurons [140–144].

Combining these data and the proprioceptive driving data gives the following hypothesis for cardiac rhythmicity in vivo (fig. 4d). The tension induced by the previous heartbeat hyperpolarizes the large and small cells due to mechanosensitive conductances in their dendrites. This hyperpolarization induces postinhibitory rebound in the neurons, and this, possibly in combination with a small-cell depolarizing pacemaker current, results in the small cells depolarizing above driver potential threshold and firing a burst. The postinhibitory rebound also induces a driver potential in the large cells, and these driver potentials passively conduct toward the distant spike initiation zones of the large cells. The combined small-cell excitatory input and the decremented large-cell driver potentials drive the large-cell spike initiation zones above threshold. Large-cell spikes induce another heart contraction, and the cycle repeats.

Higher-Order Control of the Cardiac Rhythm

Three extrinsic neurons, two excitors and one inhibitor, control the cardiac rhythm [145]. The inhibitor produces discrete inhibitory postsynaptic potentials, and the excitors a slow depolarization, in the large cells. One excitor contains tyrosine hydroxylase [137], implying the presence of dopamine, and dopamine accelerates the heart rhythm. A candidate transmitter for the remaining excitor is acetylcholine, which also excites the heart [146]. The pericardial organs, which contain 5-HT, dopamine, and octopamine and the peptides proctolin, CCAP, and FMRFamide-like peptides, all of which change heart rate and increase spike frequency, burst spike number, and burst duration [147–155], may be a source of neurohemal modulation for the heart.

Swimmeret System

Overview

Swimmerets are paired appendages (2 per segment) located on the ventral side of 4 adjacent abdominal segments. Swimmeret beating aids forward swimming, burrow ventilation, egg ventilation in gravid females, and postural control. The swimmerets of each segment beat in phase; power and return motor neurons fire in strict antiphase (fig. 4e). Swimmerets in adjacent segments beat with a fixed anterior-posterior phase relationship that results in a posterior to anterior metachronal wave of beating along the body axis (fig. 4f) [156]. The swimmeret system was the first example of both a centrally generated motor pattern [10, 156–158] and of 'command' neurons – neurons that start and stop CPG rhythmicity [159]. The system has more recently served as an experimental and computational model for investigating coupled oscillator networks (phase-locked CPGs that coordinate the activity of multiple body segments).

Cellular Basis of Pattern Generation within a Single Segment

If the intersegmental connectives are cut, each ganglion generates an independent swimmeret rhythm in which the 2 swimmerets of each segment still beat in phase. When the ganglion is bisected along the midline, the 2 swimmerets continue to beat, but their activity is no longer coordinated. The swimmeret rhythm is thus generated by chains of serially repeated pairs of CPGs, one in each hemiganglion, that are interconnected both bilaterally across the midline and across body segments.

Current injection into swimmeret motor neurons resets the rhythm, and it was originally proposed that the motor neurons were an important part of the CPG [160]. It has since been shown that the synaptic connections among the motor neurons, and from them to the CPGis, are weak and that the motor neurons are not required for normal CPG activity [161]. The actual CPG is composed of interneurons. Eight interneurons that alter motor neuron activity are present in each hemiganglion. Current injection into 4 of these can reset the rhythm (which are thus presumably members of the CPG). The synaptic interconnections of the CPG neurons have not been experimentally determined by paired neuron recordings, but the hypothetical model shown in figure 4g (dashed box) is consistent with observed interneuron activity. Current injection into the other 4 interneurons changes motor neuron firing strength, but cannot reset the rhythm. Two of these neurons oscillate with the CPG (the activity

of the others is unknown). None of the interneurons are spiking, and thus the entire 8-neuron ensemble functions via graded synaptic transmission alone.

Swimmeret motor neurons use GABA [162, 163] and glutamate [164, 165] as transmitters, and are inhibited by GABA and glutamate [161]. All CPGi to motor neuron connections are inhibitory, and it is therefore tempting to speculate that the CPGis use GABA or glutamate as transmitters. However, picrotoxin (which blocks GABAergic synapses in this system) does not abolish CPG rhythmicity [161]. The basis of motor neuron firing is unknown. Two possibilities [166] are tonic excitatory drive to the motor neurons (from unknown sources) which inhibitory input from the CPG transforms into bursts and motor neuron endogenous properties (e.g. postinhibitory rebound and plateau potentials), as is seen in the stomatogastric, ventilatory, and cardiac ganglion systems.

With respect to intrasegmental coordination of the 2 swimmerets, there are 5 bilaterally projecting interneurons in each ganglion, 2 of which are spiking [167]. Interneurons 1A and 1B receive discrete, presumably action-potential-induced, postsynaptic potentials in phase with the coupled CPG activity, and TTX (which blocks action potential production) uncouples the activity of the 2 swimmerets [168]. These data suggest that at least 1 of the bilaterally projecting spiking interneurons coordinates the 2 swimmeret CPGs of each segment.

Intersegmental Coordination

Three bilaterally symmetrical, segmentally repeated interneurons mediate intersegmental coordination [169, 170]. Recordings from coordinating axons in the interganglionic connectives, and experiments in which coupling was maintained between nonneighboring ganglia when synaptic transmission in single intervening ganglia was blocked with low Ca^{2+}/high Mg^{2+} saline, show that the intersegmental coordinating interneurons extend at least 2 ganglia from their ganglion of origin [171, 172]. TTX abolishes intersegmental coordination, which thus depends on action potentials. CPGi oscillations are also more variable in TTX, suggesting that intersegmental or bilateral (since 2 of the unisegmental bilateral interneurons are also spiking) connections refine or stabilize the motor pattern via spike-mediated timing signals.

Metachronal phase coupling is maintained in two ganglia chains [173]. The synaptic connectivity from the CPGis (and/or motor neurons) onto the intersegmental coordinating interneurons, and from the intersegmental coordinating interneurons onto CPGis (and/or motor neurons), have not been experimentally determined. Theoretical work with coupled oscillator chains suggests that one way a metachronal wave can arise is if there is an anterior to posterior increase in the inherent cycle periods of the individual oscillators of the chain. However, the cycle period of isolated segment CPGs does not increase from anterior to posterior segments, nor does altering anterior ganglion CPG cycle period alter the metachronal wave [174, 175]. This mechanism is thus unlikely to underlie the observed intersegmental coordination. An alternative mechanism is based on the observation that ascending intersegmental coordination interneurons fire in phase with interneuron 2A, and descending intersegmental coordination interneurons with interneuron 1B. Comparison of modeling and experimental data [176, 177] suggests that patterns in figure 4g, in which ascending interneurons inhibit interneuron 1A and excite interneuron 1B, and descending interneurons inhibit interneuron 1A and either inhibit interneuron 2A or excite interneuron 1B, best fit the data.

Extrinsic Control of the Swimmeret System

Descending interneurons can start the swimmeret rhythm and alter its frequency [108, 159, 178, 179]. Immunohistochemical work and comparison of interneuron stimulation and modulator application shows that these inputs use at least proctolin (excitation) and octopamine (inhibition) [180–183]. Serotonin [182], dopamine [184], the cholinergic agonists pilocarpine and nicotine [185], and the peptide CCAP also modulate swimmeret activity; CCAP-like immunoreactivity is present in the region of the abdominal ganglia where the CPGis are located [186].

Proprioceptive Feedback

Cuticular receptors, strain-sensitive hypodermal mechanoreceptors, setae, and hairs are present on the abdomen and respond to swimmeret and water movements [187–190]. Feedback from these sources is not required for individual CPG activity or bilateral and intersegmental coordination, but maintaining these feedback loops amplifies and reinforces system activity [187], and some [191], but not all [192], can entrain the rhythm. The feedback is rapid enough that cycle-by-cycle modification of the rhythm is possible [188]. Proprioceptive feedback may also play a role in maintaining intersegmental coordination [193]. As in the ventilatory system, several sensory neurons are nonspiking [191, 192] and receive cyclic input in phase with central activity that may serve to modulate or gate sensory feedback.

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Review

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Feeding Neural Networks in the Mollusc *Aplysia*

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Central pattern generator · Invertebrate · Command-like neurons · Feeding

Abstract
Aplysia feeding is striking in that it is executed with a great deal of plasticity. At least in part, this flexibility is a result of the organization of the feeding neural network. To illustrate this, we primarily discuss motor programs triggered via stimulation of the command-like cerebral-buccal interneuron 2 (CBI-2). CBI-2 is interesting in that it can generate motor programs that serve opposing functions, i.e., programs can be ingestive or egestive. When programs are egestive, radula-closing motor neurons are activated during the protraction phase of the motor program. When programs are ingestive, radula-closing motor neurons are activated during retraction. When motor programs change in nature, activity in the radula-closing circuitry is altered. Thus, CBI-2 stimulation stereotypically activates the protraction and retraction circuitry, with protraction being generated first, and retraction immediately thereafter. In contrast, radula-closing motor neurons can be activated during either protraction or retraction. Which will occur is determined by whether other cerebral and buccal neurons are recruited, e.g. radula-closing motor neurons tend to be activated during retraction if a second CBI, CBI-3, is recruited. Fundamentally different motor programs are, therefore, generated because CBI-2 activates some interneurons in a stereotypic manner and other interneurons in a variable manner.

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Introduction

Although relatively simple neural networks mediate feeding behaviors in *Aplysia,* these networks exhibit a great deal of flexibility. For example, feeding is affected by motivational states [1–20], and can be subjected to operant and classical conditioning [21–32]. *Aplysia* feeding has, therefore, proven to be unusually experimentally advantageous for studies of behavioral plasticity.

Initially, *Aplysia* feeding was primarily studied with a top-down approach, i.e., investigators started with behavior then proceeded to circuit analyses [33]. Consequently, the characterization of the *Aplysia* feeding circuitry is still very much ongoing, and it has not been described in detail in a review article. However, see Kupfermann et al. [18] and Kupfermann [34] for reviews of the modulation of feeding and the generation of behavioral states, and

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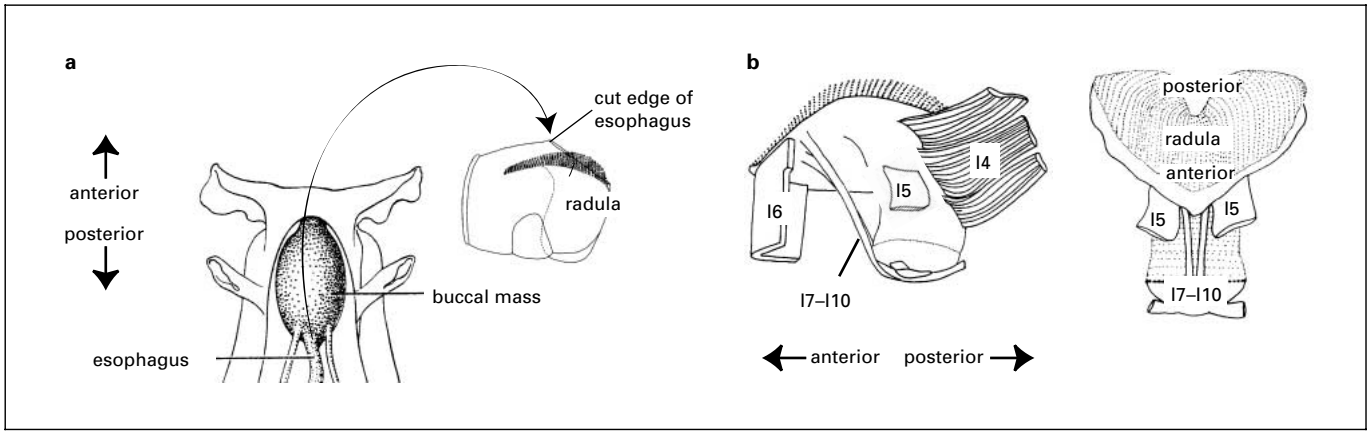

Fig. 1. The radula and attached musculature in *Aplysia*. **a** Radula and buccal mass position in the head of an *Aplysia*. Left: dorsal view of the head of an *Aplysia* in which the skin overlying the buccal mass has been removed [106]. Right: side view of a buccal mass illustrating the relative position of the radula. In part, the outline of the radula is dotted to indicate that these regions are not normally visible through the musculature of the buccal mass [86]. **b** Radula and attached musculature. Left: a side view of the radula with some of its directly attached musculature after most of the buccal mass has been removed. Labeled are intrinsic muscles I4, I5, I6, and the I7–I10 complex [86]. Right: a rear view of the radula and attached musculature, which illustrates that the radula is bilaterally symmetrical.

Elliott and Susswein [33] for a review of the comparative neuroethology of feeding control in molluscs. This review differs in that it specifically focuses on *Aplysia* circuitry (i.e., comparative issues are for the most part not discussed), and the emphasis is on basic mechanisms for pattern generation (as opposed to modulation and plasticity).

Feeding Behaviors Most Extensively Studied at the Circuit Level

Although feeding in *Aplysia* consists of both appetitive and consummatory phases, consummatory feeding has been most extensively characterized at the circuit level [for studies of appetitive behavior, see ref. 19, 21–27, 35–43]. More specifically still, a number of studies have focused on movements of the odontophore and radula. The radula is a sheet of semi-hardened tissue covered with rows of chitinous teeth that is used to grasp food and pull it into the buccal cavity (fig. 1a, b) [44].

The radula is bilaterally symmetrical, and consists of two halves (fig. 1b, right) [45]. A number of muscles are directly attached to the radula halves. In general, these muscles are intrinsic to the buccal mass and are therefore referred to with an 'I'. Extrinsic muscles are given the designation 'E' [13, 44, 46]. When some radula muscles contract, the radula halves are pulled apart, i.e., the radula is 'opened' (fig. 1b, right; 2b). When other muscles contract, the radula halves are brought together, i.e., the radula is 'closed' (fig. 2b). Additionally, the radula can move towards the jaws and towards the esophagus (fig. 2). Often these movements are referred to as protraction and retraction, although they are actually more complex [1, 47, 48].

Three types of consummatory feeding responses have been most extensively characterized; bites, swallows, and rejection movements (fig. 2b) [45]. Bites and swallows are ingestive, i.e., radula opening and protraction occur more or less (but not completely) simultaneously, as do radula closing and retraction (fig. 2b, c). Bites occur when animals make ingestive responses but do not successfully grasp food (fig. 2b) [45]. The radula protracts open, and then retracts closed to return to a neutral state [1]. Bites are converted to bite-swallows when food is ingested [45]. Under these conditions, the radula closing and retraction phase of behavior is enhanced and prolonged so that food will be deposited in the esophagus (fig. 2c) [49]. The enhanced radula retraction that occurs during a swallow is often referred to as hyperretraction. Rejection responses are egestive; radula closing and protraction occur more or less (but again not completely) simultaneously, as do radula opening and retraction (fig. 2d). This combination of radula movements will tend to push an object out of the buccal cavity.

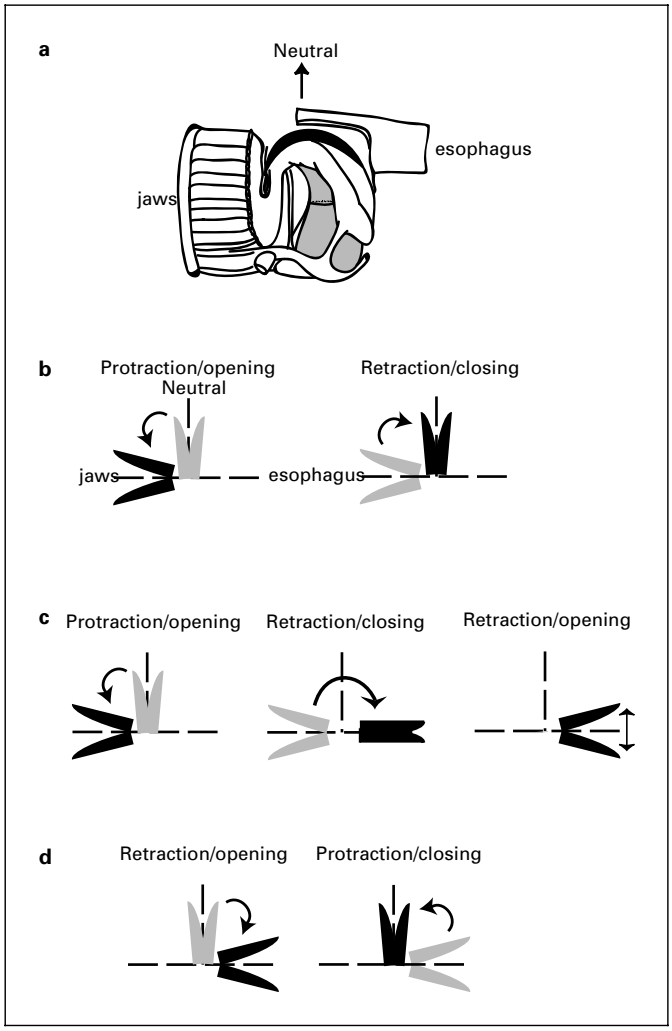

Fig. 2. Radula movements during consummatory feeding responses. **a** Radula in neutral position. Drawing of a partially dissected preparation, which indicates the resting (neutral) position of the radula within the buccal mass. The radula is shown in gray and black. **b–d** Schematic drawings illustrating radula movements during feeding. In each schematic, gray indicates the initial position of the radula and black represents the final position. **b** Bite. When *Aplysia* bite, the radula opens and protracts (left) and then closes and retracts (right) to return to the initial (i.e., neutral) position. **c** Bite-swallow. When a bite-swallow is generated, the radula opens and protracts as during a bite (left). Food contact is then detected, and the closing/retraction phase of behavior is enhanced so that food can be pulled into the buccal cavity and deposited in the esophagus (middle). The radula then opens so that food will be released into the esophagus (right). **d** Egestive response. When animals generate egestive responses, phase relationships between radula opening vs. closing and protraction vs. retraction are changed. The radula retracts open (left) and protracts closed (right).

Other consummatory responses that have been described, albeit less extensively, include swallow/tears [47, 50] or cuts [51]. Cuts or tears can be triggered if *Aplysia* are fed strips of food and a sufficient counterweight is attached [51]. Cutting releases food, presumably to prevent it from being pulled out of the buccal cavity [51]. Additionally, *Aplysia* also appear to be capable of making grazing movements in which animals locomote with their mouths against a substrate while rhythmic radula movements occur [52].

To summarize, bites, swallows, and rejection movements have been most extensively characterized in *Aplysia.* Consequently, most studies of feeding motor programs triggered in the isolated nervous system interpret data with respect to these behaviors. As other behaviors are described, however, it may be necessary to refine or reevaluate some of the current classifications of rhythmic activity.

Circuitry for Ingestive Radula Movements: Biting

The circuitry that mediates feeding in *Aplysia* is located in two ganglia, the cerebral ganglion and the buccal ganglion (fig. 3a). The buccal ganglion is clearly necessary for all consummatory behaviors [53] since it contains the motor neurons that innervate the feeding musculature [54, 55]. A more controversial question has been whether the cerebral ganglion is essential. In part, the role of the cerebral ganglion appears to be behavior dependent. For biting, the cerebral ganglion does appear to be necessary. Biting responses cannot be triggered if the connection between the cerebral and buccal ganglia (the cerebral-buccal connective) is lesioned or crushed [53, 56–59].

Biting is presumably initiated when cerebral-buccal interneurons (CBIs) are activated (fig. 3a) [60]. These neurons are referred to as CBIs because they have somata in the cerebral ganglion and they project to the buccal ganglion. Currently, approximately 13 CBIs have been identified [61]. A number of CBIs are activated by food-related stimuli (although, as might be expected, this activation is indirect) [60, 62]. In general, the CBIs make synaptic connections with both buccal interneurons and buccal motor neurons (fig. 3a). Thus, buccal motor neurons receive input from the CBIs both directly and indirectly (via buccal interneurons). Buccal motor neurons in turn innervate the feeding muscles, which for the most part are nonspiking (fig. 3a). The total number of motor neuron action potentials in general determines contraction amplitude

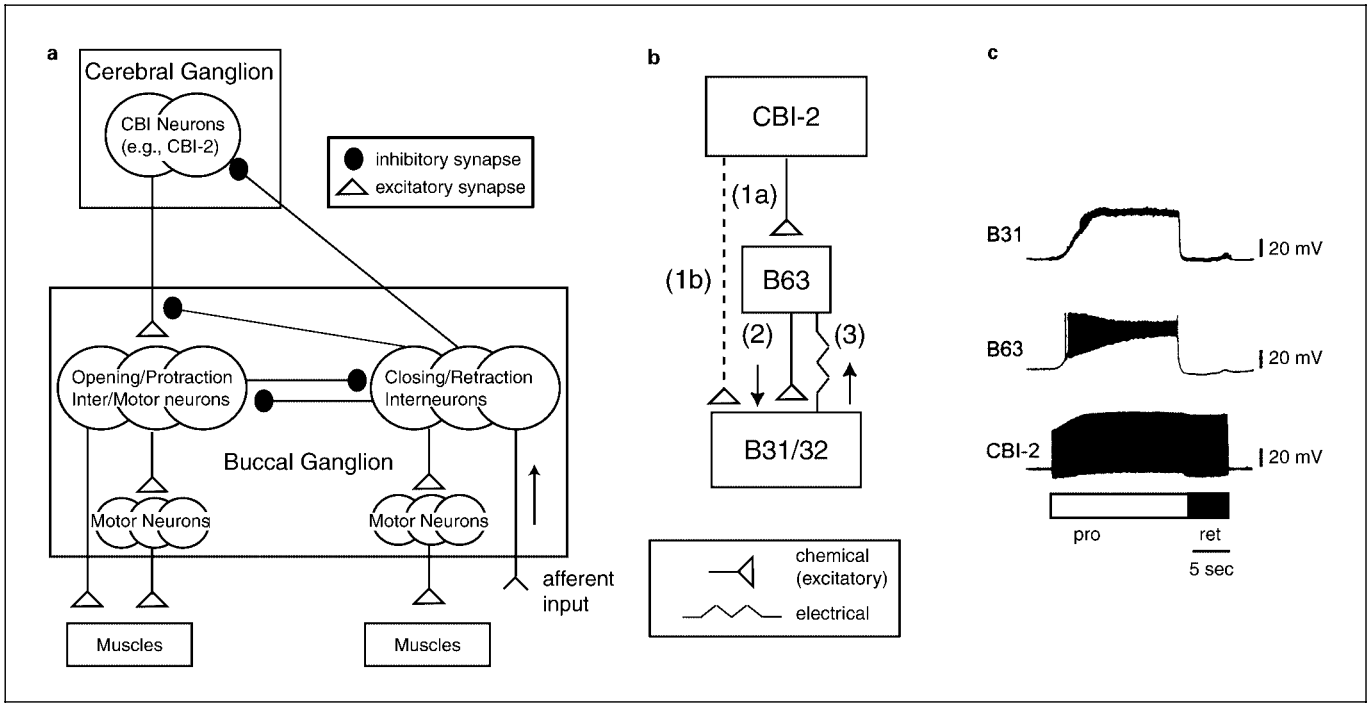

Fig. 3. a Schematic representation of the circuitry that generates ingestive activity. Motor programs can be triggered via activation of CBIs (notably CBI-2). CBI-2 makes fast excitatory connections with protraction interneurons (some of which are also motor neurons). The protraction circuitry makes inhibitory connections with the retraction circuitry. The retraction circuitry makes inhibitory connections with the protraction circuitry and with CBI-2. Inhibitory connections with CBI-2 are made in both the buccal ganglion and the cerebral ganglion. Some closing/retraction interneurons have peripheral process and dual function, i.e., they also function as afferents. **b** Excitatory connections between CBI-2 and the protraction neurons B63, and B31/B32. CBI-2 excites both B63 and B31/B32, but the direct connection with B31/B32 is relatively weak (indicated by the dashed line). B63 makes a chemical excitatory connection with the B31/B32 neurons and is electrically coupled to these cells. During the protraction phase of motor programs, a positive feedback loop is created in that B63 excites B31/B32, which then re-excite B63. **c** CBI-2-induced excitation of B63 and one of the B31/B32 neurons. Note that B63 is excited first. Also note the unusually large sustained depolarization in the B31/B32 neuron (according to Hurwitz et al. [70]). pro = Protraction; ret = retraction.

during a burst of activity, i.e., motor neuron firing frequency and duration are both important [63].

One CBI that has been implicated in the generation of biting is the cell CBI-2 [60]. It should be noted, however, that subsequent to the characterization of CBI-2, a second CBI (CBI-12) was identified that has morphological features that are similar to those of CBI-2 [43, 64]. CBI-2 and CBI-12 are not electrically coupled, and activation of one cell does not recruit the other. Nevertheless, motor programs triggered by the two neurons are similar [43, 64]. Because the two CBIs are so alike, it is possible that they have, in some cases, been confused. This is particularly likely to have been true in studies that were conducted prior to the 1999 characterization of CBI-12. Criteria that can be used to distinguish one neuron from the other have now been described [43, 65].

Under physiological conditions, biting motor programs are presumably triggered when CBI-2 is activated by afferent input [60]. Experimentally, however, CBI-2 motor programs are triggered via injection of depolarizing current, i.e., via brief current pulses (fig. 3c, 4) or via injection of direct current (fig. 5). Additionally, CBI-2 can be pharmacologically activated if the nonhydrolyzable cholinergic agonist, carbachol, is applied to the cerebral ganglion [66]. Radula movements induced by carbachol are also primarily biting-like [66]. It has been noted, however, that CBI-2 is not the only CBI activated by carbachol, and that carbachol- and CBI-2 induced motor programs are similar but not identical.

To summarize, biting responses in intact animals are most likely generated when the command-like neuron CBI-2 is activated. Experimentally, fictive biting motor

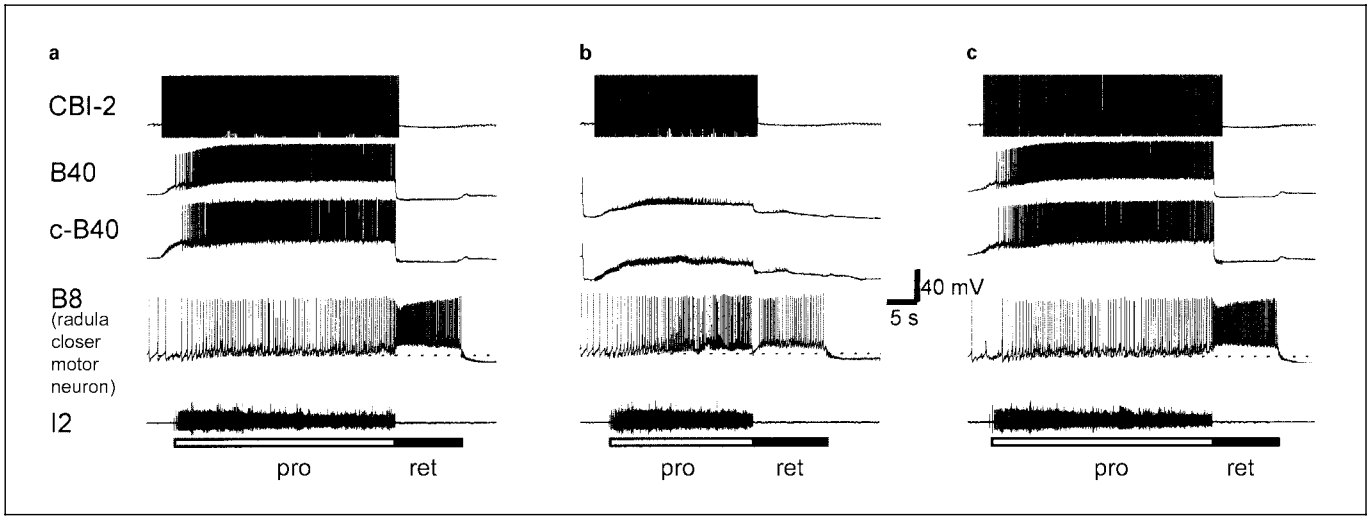

Fig. 4. Interneuron B40 is important for expression of CBI-2-elicited ingestive motor programs. **a–c** Single cycles of feeding motor programs induced by stimulation of CBI-2 with brief current pulses (top traces). Cycles of activity were approximately 2 min apart. The open and filled bars indicate the protraction and retraction phases of activity, respectively (bottom line). In all three cases, CBI-2 stimulation was maintained throughout protraction, but was then terminated when retraction was initiated. **a** A cycle of ingestive-like activity (the radula closer motor B8 is predominantly active during the retraction phase of the motor program). **b** The buccal interneuron B40 was bilaterally hyperpolarized. This converted ingestive-like activity to an intermediate program (there is increased activity in B8 during protraction). **c** The B40 hyperpolarization was relieved and the program was again ingestive-like (according to Jing and Weiss [67]). pro = Protraction; ret = retraction.

programs can be generated in the isolated nervous system via stimulation of CBI-2, or by applying carbachol to the cerebral ganglion. Below, we primarily focus on motor programs triggered by CBI-2 because they have been more extensively characterized than carbachol-induced programs.

CBI-2 Elicited Motor Programs

CBI-2 motor programs consist of at least two phases, a radula protraction phase followed by a radula retraction phase (fig. 4, 5). Additionally neurons are activated that produce radula opening and closing movements. To a large extent, the interneurons that generate protraction vs. retraction appear to be different from those that generate opening vs. closing (fig. 6) [67]. Below, we begin by describing the circuitry that generates protraction vs. retraction.

Generation of Radula Protraction and Retraction

When rhythmic activity is triggered via CBI-2, the radula protraction phase of the motor program is always initiated first (fig. 4, 5) [68]. This is a result of the fact that

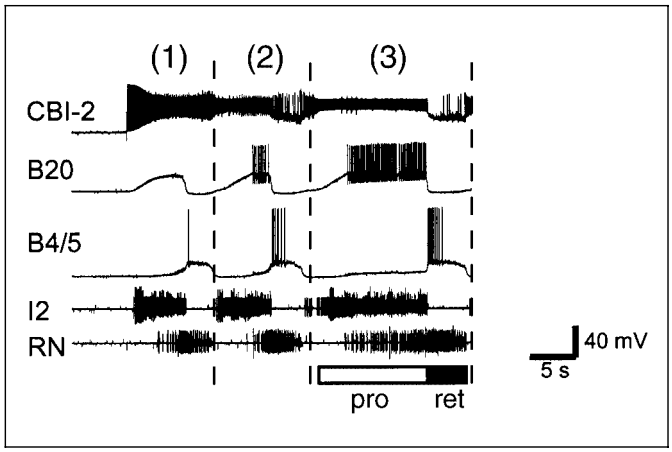

Fig. 5. Three cycles of a motor program induced by continuous injection of direct current into CBI-2. Dashed lines delineate each cycle of the program. The protraction and retraction phases of activity are indicated for the third cycle by the open and filled bars (bottom line). Note the variability in the evoked activity (according to Jing and Weiss [65]). RN = Radula nerve; pro = protraction; ret = retraction.

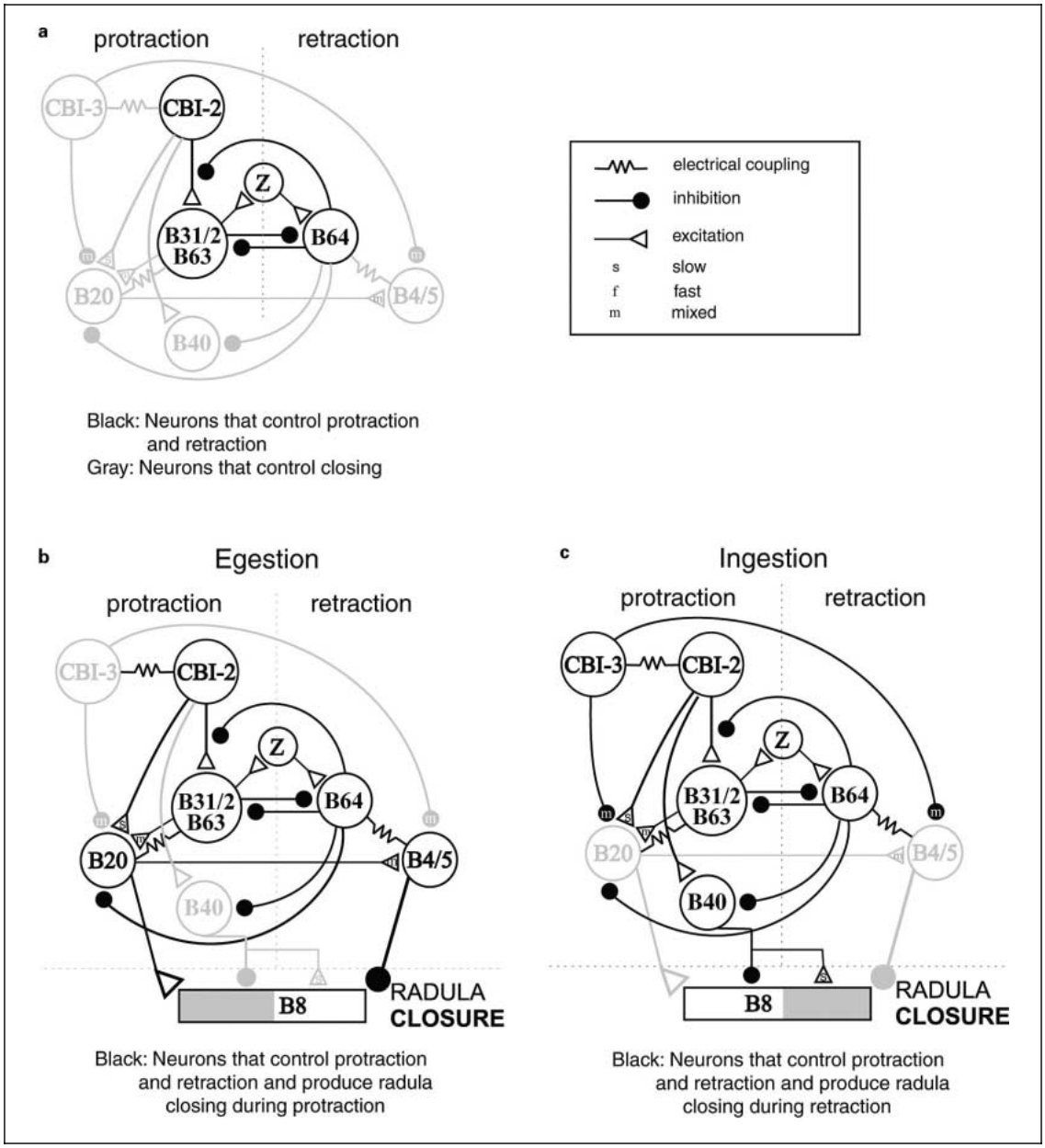

Fig. 6. Interneurons activated during CBI-2-evoked motor programs. **a** Interneurons that are primarily important for the generation of protraction and retraction are shown in black. Protraction is initiated when CBI-2 excites interneurons such as B63, and interneurons/motor neurons such as B31/B32. During retraction, the protraction circuitry and CBI-2 are inhibited by neurons such as B64. B64 is also electrically coupled to motor neurons that produce radula retraction (not shown). It is currently not clear how protraction/retraction phase transitions are triggered, but the activation of an unidentified neuron (the z cell) may be involved (see text). Gray: neurons that control closing. **b**, **c** Two classes of neurons are shown in black: (1) neurons that are important for the generation of protraction and retraction, and (2) interneurons that are primarily important for the control of the B8 radula closer motor neurons. During CBI-2 motor programs, the B8 neurons can be activated either during protraction, which makes activity egestive-like (**b**), or during retraction, which makes activity ingestive-like (**c**). **b** When activity is egestive-like, CBI-3 is generally not recruited by CBI-2. B20 is, however, activated during the protraction phase of the motor program. B20 directly excites B8. Additionally, B20 makes a slow excitatory connection with the B4/5 neurons and these cells are activated during the retraction phase of the motor program. The B4/5 neurons make a fast inhibitory connection with B8. **c** When activity is ingestive-like, CBI-3 is generally recruited by CBI-2. CBI-3 inhibits B20 activity during protraction and B4/5 activity during retraction. Additionally, a cell not active during egestive-like motor programs (B40) is activated. B40 inhibits B8 activity during protraction (via fast inhibition) and promotes B8 activity during retraction (via slow excitation) [65].

CBI-2 makes monosynaptic excitatory connections with a number of protraction interneurons and motor neurons (fig. 3a, b) [64, 69, 70]. Neurons that have been particularly well studied in this context include a group of buccal cells that are thought to be important for generating protraction movements, i.e., the neurons B63, B31, and B32 (fig. 3b) [71–75]. B31 and B32 are electrically coupled, and are virtually indistinguishable [72]. Therefore, they are often referred to together as B31/B32. B63 is electrically coupled to B31/B32, and, in addition to the electrical connection, there is a strong excitatory chemical connection from B63 to the contralateral B31/B32 neurons [71]. B63 and B31/B32 have been described as a functional unit since sufficient depolarization of one of these cells excites all the others [71, 73]. There is a direct relationship between B63/B31/B32 activity and protraction movements since B31/B32 are motor neurons [74, 75]. Specifically, they innervate the I2, which is a major protractor muscle [75]. Thus, protraction is initiated when CBI-2 excites motor/interneurons like B63/B31/B32 (fig. 6a).

If CBI-2 is sufficiently activated, a brief period of stimulation will induce a protraction phase of a motor program that persists after CBI-2 stops spiking (fig. 4). (This is also true with feeding behavior, i.e., once a feeding response is initiated, it is generally completed even if food is removed [45].) In part, the persistence of activity in B63/B31/B32 is likely to be due to the fact that a positive feedback loop is created in this circuitry (fig. 3b) [71]. Thus, during CBI-2-elicited motor programs, B63 is activated first (fig. 3c, 7) [71]. B63 provides excitatory input to the B31/B32 neurons via electrical and facilitating chemical excitation (fig. 3b, step 2) [70, 71]. The B63-induced excitation of B31/B32 is essential for the generation of motor programs, i.e., if B63 is hyperpolarized, programs are not triggered [70]. Presumably, this is because direct connections between CBI-2 and B31/B32 are relatively weak (fig. 3b, step 1b), and in B31/B32, the threshold for excitation is relatively high [70]. As B31/B32 are depolarized, this depolarization is transmitted back to B63 via the electrical coupling, i.e., the loop is closed (fig. 3b, step 3) [71, 73]. Transmission in the B31/B32-to-B63 direction is likely to be facilitated by a number of factors [73]. For example, the B31/B32:B63 coupling ratio is asymmetric (favoring the B31/B32-to-B63 direction). Sustained depolarizations in B31/B32 are also unusually large (i.e., 30–40 mV) (fig. 3c). Presumably, the large depolarizations result from the unusual biophysical properties of the B31/B32 neurons. Namely, plateau or plateau-like potentials are observed in B31/B32, but the somata of these cells do not generate action potentials [72–74].

To summarize, with sufficient activation of the command-like neuron CBI-2, a sustained depolarization is generated in the protraction motor neurons B31/B32. Consequently, if CBI-2 stimulation ceases, B31/B32 do not immediately repolarize. This sustained depolarization may in part result from the fact that a positive feedback loop is created in the protraction circuitry. Additionally B31/B32 generate plateau or plateau-like potentials.

B63 and B31/B32 are not the only neurons active during the protraction phase of CBI-2-induced motor programs. Some of the other cells activated are important for determining whether programs are ingestive-like or egestive-like (as is described below). Still other cells can alter the onset of activity in B31/B32. B34 [71] is a cell in the latter category (fig. 7) [70]. Thus, B34 is directly excited by CBI-2 and is reliably recruited when CBI-2 is activated (recruitment in other motor programs, however, does not always occur) [65, 70]. Although B34 provides excitatory input to B31/B32, hyperpolarization of B34 does not block CBI-2-induced activation of B31/B32 (presumably because the CBI-2-B63-B31/B32 pathway is utilized) [67, 70]. However, the slope of the depolarization in B31/B32 is decreased, i.e., B31/B32 depolarize more slowly. Thus, B34 is not essential for protraction, but it does modify its expression.

Parametric features of protraction can also be affected by the recruitment of another buccal interneuron, B50 [76]. B50 has been of particular interest since it appears to be homologous to a well-characterized interneuron that can trigger rhythmic activity in *Lymnaea,* the slow oscillator [77, 78]. (To call attention to the homology, the *Aplysia* neuron was given a designation that resembles the *Lymnaea* designation, i.e., '50' vs. 'SO'.) B50 makes excitatory connections with B34 and with the B63/B31/B32 cells [76]. B50 differs from CBI-2 in that direct connections from B50 to B31/B32 are relatively strong [76]. (As discussed above, direct connections between CBI-2 and B31/B32 are relatively weak.) Although CBI-2 directly excites B50, this connection is not strong enough for B50 to be recruited every time a motor program is generated. When B50 is activated, however, parametric features of CBI-2-triggered motor programs are altered, e.g. the protraction phase of the motor program is shortened [76].

While protraction is ongoing, retraction interneurons and motor neurons are inhibited (fig. 3a, 6a). In part, this inhibitory input arises from the interneurons that provide excitatory input to protraction motor neurons; for example, B63 makes a weak inhibitory connection with a retraction interneuron B64 [71, 79] and B34 inhibits B64 [80]. Additionally, however, other cells, i.e. the B52 neu-

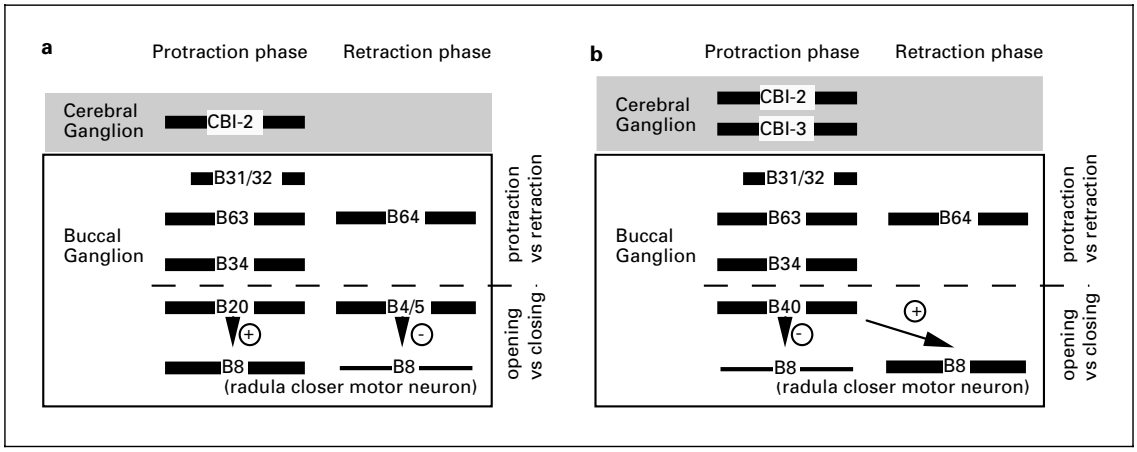

Fig. 7. Schematic illustration of phase relationships between neurons active during egestive (**a**) and ingestive (**b**) motor programs generated by stimulation of CBI-2. The CBI-2 program is represented as a two-phase program; i.e., as a radula protraction phase followed by a radula retraction phase. Activity of neurons in the cerebral ganglion is represented within the gray area. Activity of neurons in the buccal ganglion is indicated in the white boxes. The buccal circuitry is also subdivided. Activity of neurons that primarily generate protraction vs. retraction movements is represented above the dotted line. Activity of neurons that primarily generate opening vs. closing movements is represented below the dotted line. The thin line used to represent B8 activity indicates that activity is weak. + = Excitation; – = inhibition.

rons, are also activated (at least during carbachol-induced motor programs [81, 82]). The B52 neurons make strong inhibitory connections with a number of elements of the retraction circuitry [81–83]. Direct excitatory connections between the B52 neurons and protraction motor neurons have, however, not been described. Moreover, B52 stimulation does not trigger rhythmic activity [83]. At least to some extent, therefore, the generation of protraction and the inhibition of retraction may be mediated by different neurons.

To summarize, a number of buccal interneurons activated during the protraction phase of CBI-2-elicited motor programs have been identified. Some of these neurons appear to be essential for generating protraction movements (i.e., B31/B32 and B63). Others (a) modify parametric features of protraction (i.e., B34), (b) inhibit the retraction circuitry (i.e., B52), and (c) determine activity in the opening vs. closing circuitry (i.e., B20 and B40, which are discussed below).

The protraction phase of a CBI-2-elicited motor program is immediately followed by the retraction phase (fig. 4, 5). The termination of protraction appears to be an active process, e.g. protraction neurons are hyperpolarized below resting membrane potential during retraction [79]. At least in part, this inhibitory input is provided by the retraction interneuron B64 (fig. 6a) [79, 84]. Inhibitory input from B64 is highly effective, i.e., B64 stimulation can phase advance retraction [79, 84]. It is not, however, currently clear how activity in B64 (or the rest of the retraction circuitry) is triggered, i.e., it is not clear what makes protraction-retraction phase transitions occur. In quiescent preparations, B64 does not show postinhibitory rebound excitation at its resting membrane potential.

It has been suggested that B64 activation may be triggered by an as yet uncharacterized circuit element, which has been referred to as the 'z' cell (fig. 6a) [85]. The z cell would be activated towards the end of protraction and would provide excitatory input to B64, which would inhibit the protraction circuitry [85]. B64 also makes electrical connections with other retraction neurons. Consequently, it could play a role in driving retraction. Additionally and/or alternatively, some of the characterized circuit elements active during protraction may provide slow excitatory input to retraction neurons [84]. Phase transitions may, therefore, be determined by the balance of excitatory and inhibitory inputs received by the retraction circuitry during the protraction phase of the motor program.

At least two of the interneurons that are depolarized during the retraction phase of CBI-2 motor programs have plateau or plateau-like potentials, i.e. B64 [79] and a second interneuron B51 [83]. B64 and B51 differ in that,

although both cells are depolarized, B51 has a higher threshold for plateau initiation and most commonly does not spike during CBI-2 (or carbachol)-elicited motor programs [86]. The plateau properties of B51 may, however, contribute to the generation of retraction movements since B51 is electrically coupled to B64 and to a number of retraction motor neurons [79, 83, 86]. Thus, a number of the characterized connections in the retraction circuitry are electrical.

While retraction is ongoing, neurons that generate protraction movements are inhibited (fig. 3a, 6a) [79]. Additionally, CBI-2 receives inhibitory input during retraction (fig. 3a, 6). In part, CBI-2 is inhibited by neurons that are referred to as buccal-cerebral interneurons (BCIs) [43, 87]. These cells have somata in the buccal ganglion but project to the cerebral ganglion via the cerebral-buccal connective. One such neuron is B19 [43]. B19 is not, however, always driven to spike during CBI-2-elicited motor programs, and somatic inhibition of CBI-2 is not always observed. CBI-2 is, however, also presynaptically inhibited by B64 in the buccal ganglion (fig. 6a) [84]. B64 is always activated during CBI-2-generated motor programs [70, 80, 84]. Consequently, although somatic spiking can sometimes be observed in CBI-2 during the retraction phase of motor programs, this activity is not transmitted to the protraction circuitry in the buccal ganglion [84].

To summarize, during the retraction phase of motor programs, interneurons with plateau-like potentials are activated (e.g. B64). B64 inhibits protraction interneurons and presynaptically inhibits CBI-2. Consequently, the protraction phase of the motor program is terminated.

Some molluscs (e.g. *Lymnaea* and *Helisoma*) have been described as having a three-phase motor program, protraction, retraction, and hyperretraction or swallowing [88, 89]. During CBI-2-elicited motor programs in *Aplysia,* activity is observed in buccal neurons immediately after the retraction phase of the motor program. It is unlikely, however, that this activity corresponds to the hyperretraction or swallow phase described elsewhere. For example, immediately after retraction, activity is observed in the B52 neurons [81]. The B52 neurons make inhibitory connections with the retraction circuitry, so they certainly do not initiate retraction movements. On the contrary, it has been suggested that B52 activity is important for the termination of retraction [82]. Other cells that can be depolarized immediately after retraction are radula-opening motor neurons, e.g. B48 and B66 [90, 91]. Postretraction activity in B48 and B66 is, however, not always very pronounced when programs are triggered by CBI-2 [90, 91]. Presumably, this is due to the fact that CBI-2-induced programs are generally biting-like and radula opening at the peak of retraction is not important since food is not ingested. Thus, although there is activity in the buccal circuitry immediately after the retraction phase of CBI-2-elicited motor programs, it is not clear whether this activity will generate functional movements.

CBI-2-induced programs are, therefore, different from the three-phase programs observed in other molluscs, and are generally referred to as two-phase programs, because phases have been traditionally equated with movements, and a movement has not been described for the neural activity that immediately follows retraction.

When motor programs are triggered via stimulation of CBI-2, a single burst of action potentials generally elicits a single cycle of a motor program (fig. 4). For example, if CBI-2 is stimulated throughout protraction and then stimulation ceases, both protraction and retraction phases of activity are generated [62, 65, 67, 70, 80, 84]. Subsequent cycles of motor programs are, however, not necessarily observed. This suggests that the retraction circuitry does not necessarily re-excite the protraction circuitry. In general, this is similar to what has been observed when intact animals bite. Thus, when food contact is maintained, a series of bites can be triggered. If, however, food is immediately removed, it is possible to trigger a single biting response.

The fact that single cycles of CBI-2-evoked activity can be triggered has proven to be experimentally advantageous for studies that have assessed single-cell contributions to motor program generation. Thus, when single cycles of activity are generated approximately once a minute, or once every 2 min, parametric features of evoked motor programs are highly reproducible (fig. 4a vs. 4c) [62, 65, 67, 70, 80, 84]. In contrast, when repetitive activity is triggered via continuous stimulation of CBI-2, evoked activity can stabilize, but often it does not (compare cycles 1 and 3 in fig. 5). When activity is variable, it is obviously more difficult to determine whether the manipulation of a single neuron affects the ongoing motor program. Thus, the single cycle paradigm has proven to be experimentally advantageous, particularly in cases where the manipulation of a single cell alters parametric features of evoked activity but does not completely inhibit or reconfigure it.

To summarize, CBI-2-elicited motor programs are generally referred to as two-phase programs. Phase one corresponds to radula protraction and is triggered by direct excitation of neurons like the interneuron B63 and the

protraction motor neurons B31/B32. Phase two corresponds to radula retraction. It is initiated when cells like the interneuron B64 are activated, and the protraction circuitry and CBI-2 are actively inhibited. It is still not clear how protraction-to-retraction phase transitions occur.

Radula Opening and Closing during CBI-2-Elicited Motor Programs

When animals make feeding responses, the radula protracts and retracts. Additionally, the radula opens and closes (fig. 2). Above, we described the circuitry that mediates the protraction vs. retraction phases of CBI-2-elicited motor programs. Below, we will discuss the circuitry that mediates opening vs. closing. This discussion begins with a general consideration of two features of the generation of opening vs. closing that distinguish it from the generation of protraction vs. retraction. Specific connectivity in the opening vs. closing circuitry is then discussed.

Differences between Protraction vs. Retraction and Opening vs. Closing

One important difference between protraction/retraction and opening/closing is that protraction/retraction occurs in a stereotypical manner. As described above, protraction and retraction alternate, with protraction occurring first when activity is evoked by CBI-2. CBI-2-induced activation of the opening vs. closing circuitry is, however, variable in that in some cases, radula-closing motor neurons are predominantly activated during the first phase of evoked activity, i.e., during protraction (fig. 4b). In other cases, radula-closing motor neurons are predominantly activated during the second phase of evoked activity, i.e., during retraction (fig. 4a, c). As a result, CBI-2 can generate programs where the protraction and closing circuitry are simultaneously activated, or can generate programs where the protraction and opening circuitry are simultaneously activated. This is of interest since these two types of programs differ functionally. During ingestive behaviors, the radula closes during retraction (fig. 2b, c). In contrast, during egestive behaviors, the radula closes during protraction (fig. 2d).

CBI-2 is, therefore, a command-like neuron that can generate fundamentally different motor programs. At least in part, this occurs because CBI-2 triggers protraction and retraction in a stereotypical manner. Activation of the radula opening and closing circuitry is, however, variable.

Secondly, protraction vs. retraction and opening vs. closing differ in that activity in the protraction and retraction circuitry is almost completely out of phase. During CBI-2-elicited motor programs, the protraction circuitry is initially excited, and the retraction circuitry is inhibited. When retraction is initiated, the protraction circuitry becomes inactive. In contrast, during CBI-2-elicited motor programs, the radula closer motor neurons are generally active, at least to some extent during both the protraction and retraction phases of motor programs (fig. 4) [62]. (It is currently not clear whether this is also true for radula opener motor neurons.) Consequently, CBI-2-elicited motor programs are presumably not either completely 'ingestive' (e.g. 100% of the activity in radula closer motor neurons during retraction) or completely 'egestive' (e.g. 100% of the activity in radula closer motor neurons during protraction). Often, however, radula closer motor neurons are not equally active during protraction and retraction. Instead, they are predominantly active during either protraction or retraction. For example, in figure 4a and C, the radula closer motor neuron B8 is predominantly active during retraction. In contrast, in figure 4b, B8 is predominantly active during protraction. Motor programs are therefore classified as being either more ingestive-like or more egestive-like [62]. Currently, several systems are used for classifying motor programs [28, 30–32, 62, 67, 92]. Although these methods are likely to produce the same results in some cases, in other cases they may not. The method used for classifying activity can therefore affect data interpretation.

Although CBI-2 activates the protraction/retraction circuitry in a stereotypical manner, the activation of the radula-closing circuitry is highly variable. An important functional consequence of this is that programs triggered by CBI-2 can be egestive-like (radula closing during protraction), ingestive-like (radula closing during retraction), or intermediate (radula closing during both protraction and retraction).

Connectivity in the Opening/Closing Circuitry

Because the CBI-2-induced activation of the opening/closing circuitry is so functionally important, much research has gone (and is going) into determining how it occurs. Most studies to date have focused on the control of the B8 neurons, which are radula closers. A number of neurons that can be activated during CBI-2-evoked motor programs make connections with the B8 cells. All of these cells will obviously contribute to the patterning of B8 activity. Not all cells with B8 connections are, however, essential for determining whether motor programs are so altered that their classification as ingestive-like or egestive-like changes. For example, B34 is active during the

protraction phase of CBI-2 motor programs [67, 70, 71], and it makes a mixed, but predominantly excitatory connection with the B8 neurons [71]. Thus, activation of B34 will tend to make programs more egestive-like. Hyperpolarization of B34 does not, however, completely convert egestive-like motor programs to ingestive-like [67, 70]. In contrast, when other cells are depolarized or hyperpolarized, the ingestive or egestive character of the ongoing motor program is altered. For simplicity, our discussion below focuses on neurons of the latter type.

Egestive-Like Activity. When CBI-2-elicited motor programs are egestive-like, a buccal interneuron, B20 [93], is excited (fig. 6b) [65]. B20 receives input directly from CBI-2 [65] and is indirectly excited by CBI-2 via protraction interneurons, e.g. by B34, B63, and B31/B32 (fig. 6b) [65]. B20 makes a fast excitatory connection with the B8 neurons (fig. 6b, 7a) [93], which increases B8 activity during protraction [65]. Additionally, B20 provides slow excitatory input to the retraction phase neurons B4/5 (fig. 6b, 7a) [65]. These cells make monosynaptic inhibitory connections with the B8 neurons (fig. 6b, 7a) [94, 95]. During CBI-2-induced motor programs, therefore, B20 fires during protraction and produces an immediate (i.e., protraction phase) increase in B8 activity (fig. 7a). Additionally, B20 activates B4/5 with a delay, which produces a decrease in retraction phase B8 activity (fig. 7a).

The fact that B20 exerts effects on the B8 neurons via B4/5 is of interest, since the B4/5 neurons are multifunctional and innervate muscles that produce radula opening (i.e. I7–I10) [90]. The B4/5 neurons are not the primary I7–I10 motor neurons, but B4/5 activity can induce I7–I10 contractions, albeit weakly [90]. When B4/5 activity is increased, therefore, radula closing is inhibited and radula opening is simultaneously potentiated.

To summarize, when CBI-2 programs are egestive-like, the protraction interneuron B20 is excited. B20 directly excites the B8 radula closer motor neurons during protraction, and indirectly inhibits the B8 neurons during retraction (via slow effects on B4/5).

Ingestive-Like Activity. In most cases, stimulation of CBI-2 induces ingestive-like motor programs (rather than egestive-like programs). More specifically, in semi-intact preparations, evoked movements are most commonly biting-like [96]. At least in part, CBI-2-induced motor programs are likely to be ingestive-like if a second CBI, CBI-3, is recruited (fig. 6c, 7b) [62]. In the isolated nervous system, the variable nature of the recruitment of CBI-3 is in part determined by experimental technique. When CBI-2 is activated via direct current injection, CBI-3 is more apt to be recruited than in experiments where CBI-2 is activated via brief current pulses. Under physiological conditions, however, both cells are likely to be activated by peripheral stimulation. Thus, both neurons are directly activated when food is applied to the lips [60]. Presumably, therefore, CBI-2 and CBI-3 will be coactivated when food is present, which will tend to produce ingestive behavior. Interestingly, however, only CBI-2 is activated when stimuli are applied that are likely to trigger egestive behavior. Namely, stretch of the esophagus, which presumably simulates ingestion of an inappropriate substance, leads to strong inhibition of CBI-3 at a time when CBI-2 is strongly excited [62]. Presumably, therefore, when an egestive stimulus is presented, only CBI-2 will be activated.

When CBI-3 is recruited, it fires phasically during the protraction phase of CBI-2-elicited motor programs (fig. 7b). In general, CBI-3 has two types of effects. Firstly, it inhibits the egestive-like neurons that are activated by CBI-2, i.e., B20 and B4/5 (fig. 6c). Specifically, CBI-3 generates fast inhibitory postsynaptic potentials in B20, which suppresses B20 activity [65]. Additionally, CBI-3 exerts slow effects on B4/5 and decreases the excitability of these cells (fig. 6c) [65]. At least in part, this slow effect appears to be due to the release of a peptide from CBI-3, APGWamide [65]. Secondly, CBI-3 provides excitatory input to a protraction phase interneuron, B40 (fig. 6c) [80]. B40 is interesting in that it makes both fast and slow synaptic connections with the B8 radula closer motor neurons (fig. 6c, 7b) [67]. The fast connection is inhibitory. Consequently, when B40 is active during protraction, the B8 neurons are inhibited, which tends to make programs ingestive-like. In contrast, the slow connection between B40 and the B8 neurons is excitatory. Consequently, B40 drives the B8 neurons during retraction, which makes them able to fire above their spontaneous frequency.

Interestingly, B40 and CBI-3 are both GABA immunoreactive and both cells evoke inhibitory postsynaptic potentials in postsynaptic followers that can be blocked by picrotoxin [80]. When CBI-2 motor programs are triggered in the presence of picrotoxin, protraction and retraction phases of motor programs are observed as normal. B8 activity in protraction is, however, increased, which tends to make programs egestive-like [80]. Thus, GABAergic neurons appear to act together to make CBI-2-induced motor programs ingestive-like [80].

To summarize, CBI-2-induced programs are most commonly ingestive-like (presumably biting-like). When programs are ingestive-like, CBI-3 is often recruited during the protraction phase of the motor program. CBI-3 inhibits the 'egestive' neurons activated by CBI-2 (B20

and B4/5), and excites an 'ingestive' neuron, B40. B40 tends to make programs ingestive-like because it inhibits the B8 radula closer motor neurons during protraction, and excites the B8 motor neurons during retraction.

General Characteristics of Neurons That Alter the Ingestive-Like vs. Egestive-Like Nature of CBI-2-Evoked Activity. In general, neurons that most effectively alter the ingestive-like vs. egestive-like nature of CBI-2-evoked activity exert both fast and slow synaptic actions. Consequently, B8 activity during both protraction and retraction phases of motor programs is adjusted. For example, B40 makes programs ingestive-like in that it decreases B8 activity during protraction via fast inhibitory synaptic input, and increases B8 activity during retraction via slow excitatory effects (fig. 7b) [67]. In a similar vein, B20 makes programs egestive-like in that it increases B8 activity during protraction, and indirectly decreases B8 activity during retraction (via B4/5) (fig. 7a) [65]. Thus, cells that exert both fast and slow synaptic actions most effectively alter the nature of CBI-2-elicited motor programs.

A consequence of this arrangement is that protraction interneurons are utilized to generate both ingestive-like and egestive-like activity. Presumably, this is a result of the fact that it is advantageous for slow synaptic events to be initiated during protraction. In theory, a retraction interneuron could exert slow synaptic actions that are manifested during the subsequent protraction. It is possible that this is not advantageous since biting does not necessarily occur in a repetitive manner. Thus, protraction interneurons appear to play an important role in determining the ingestive-like vs. egestive-like nature of a cycle of CBI-2-elicited activity.

Additional CBIs Activated by CBI-2

As is described above, CBI-3 is often recruited by stimulation of CBI-2. Thus, a 'CBI-2-induced motor program' is in reality not simply generated by CBI-2 alone. Additional CBIs that are likely to be activated by CBI-2 include CBI-5 and CBI-6 [97]. CBI-5 and CBI-6 are electrically coupled to each other and are so physiologically and morphologically similar that they have been referred to as a single unit (i.e., as CBI-5/6) [97]. CBI-5/6 are interesting in that, although the cerebral somata appear to be capable of generating plateau or plateau-like potentials, the spike initiation site in these neurons is distant (presumably, it is in the buccal ganglion) [97]. Thus, if current is injected somatically, activity above 10 Hz generally cannot be elicited. When motor programs are triggered by CBI-2, however, buccal terminals of CBI-5/6 generate antidromic spikes at frequencies of up to 25 Hz [97]. Because the spike initiation site in CBI-5/6 is so distant, it has been difficult to experimentally manipulate these neurons. Consequently, it has been difficult to study their specific contribution to CBI-2-elicited motor programs. It appears, however, that they are phasically activated during the retraction phase of motor programs and they provide excitatory input to the retraction circuitry, e.g. to the retraction interneurons B64 and B4/5, and to the accessory radula closer motor neuron B15 [97].

Motor programs experimentally evoked by stimulating CBI-2 alone are influenced by the variable recruitment of other CBIs. Coactivation of the CBIs is also likely to occur under physiological conditions. A number of these cells respond to food-related stimuli. The specific contribution of one other CBI (CBI-3) has been examined. Contributions of other cells have not yet been evaluated.

Sensory Feedback to the Feeding Central Pattern Generator: The Bite to Bite-Swallow Transformation

Although feeding motor programs can be triggered in the isolated nervous system of *Aplysia,* sensory feedback clearly modifies rhythmic activity under physiological conditions [98]. In particular, sensory feedback is likely to be important when food is ingested, i.e., when bites are converted to bite-swallows. Under these conditions, there is a striking change in radula movements. Specifically, the radula closing/retraction phase of behavior is enhanced so that food will be pulled into the mouth and deposited in the esophagus, i.e., hyperretraction occurs (fig. 2c) [1]. Studies in intact animals have indicated that, at least in part, the enhancement of closing/retraction is mediated via a prolongation of this phase of behavior [49]. Thus, in *Aplysia,* hyperretraction does not appear to occur unless food is ingested. Hyperretractions are therefore not an integral part of all ingestive motor programs. More specifically, CBI-2-induced motor programs most commonly do not appear to have a hyperretraction phase.

In intact animals, at least two classes of sensory neurons are likely to be activated when bites are converted to bite-swallows. Some sensory neurons are radula mechanoafferents. These cells are relatively low-threshold mechanoafferents that are activated whenever anything touches the radula [99, 100]. They are, therefore, likely to be important for detecting that food has contacted the radula. The largest and best-characterized radula mechanoafferents are B21 and B22 [100]. Other sensory neurons that will be activated when food is ingested are retraction

proprioceptors [86]. The level of activity in these neurons is increased when the radula retracts, particularly when the resistance to radula retraction is increased [86]. The largest and best-characterized retraction proprioceptor is the neuron B51 [83, 86]. B21 and B51 are electrically coupled to each other, although generally not strongly enough to produce coactivation.

Interestingly, the somata of B21 and B51 are in the buccal ganglion and both cells have central sites of spike initiation [83]. Both neurons also make extensive synaptic connections with other buccal cells (fig. 3a) [83, 100]. In general, connections with protraction interneurons are inhibitory [83, 100]. Connections with interneurons and motor neurons that mediate radula closing and retraction are excitatory (i.e., are either electrical, or are chemically depolarizing) [83, 100]. Thus, B21 and B51 receive central input that can induce spiking. Additionally, these cells make synaptic connections with motor neurons and therefore can function as interneurons as well as primary afferents. Which role will predominate will presumably be determined by the nature of the motor program that is generated.

During most cycles of CBI-2- or carbachol-elicited motor programs, B21 and B51 are rhythmically depolarized during the retraction phase of the motor program. However, central input either is not sufficient to induce spiking, or low frequency activity is generated [86]. During biting-like motor programs, therefore, B21 and B51 presumably do not function as interneurons. If peripheral activation of B21 or B51 is 'mimicked', however, by inducing spiking during the retraction phase of the motor program, the duration of retraction is significantly increased [101], and retraction movements are enhanced [86]. This is observed when current is injected into a single sensory neuron [86, 101].

To summarize, during biting-like motor programs, B21 and B51 are centrally depolarized, but depolarizations are not sufficient to trigger significant activity. In an intact animal, however, B21 and B51 will presumably be peripherally activated during the retraction phase of behavior (i.e., by food contact and increased resistance to radula retraction). When this occurs, a hyperretraction will be induced, i.e., a bite will be converted to a bite-swallow.

Interestingly, experiments with B21 have shown that, although the rhythmic depolarizations during the retraction phase of CBI-2-elicited motor programs are generally subthreshold for spiking, they do play an important role in regulating (or gating) afferent transmission [91, 102]. At least in part, this appears to be due to the fact that centrally induced depolarizations regulate spike propagation in B21 [102]. Specifically, when B21 is at its resting membrane potential, spikes are not actively propagated to all output regions of the cell. Postsynaptic followers are therefore not strongly driven. In contrast, when B21 is centrally depolarized, spikes are actively propagated, and postsynaptic followers are strongly driven. Thus, during CBI-2-elicited motor programs, centrally induced depolarizations during the retraction phase of the motor program are important because they gate in peripherally generated spikes [102].

Circuitry for Egestive Radula Movements

As discussed above, *Aplysia* generate egestive as well as ingestive responses. It has been postulated that there is more than one type of egestive behavior in *Aplysia* [59, 103, 104]. In part, this is suggested by the fact that one type of response (i.e., active seaweed rejection) appears to be dependent on the cerebral ganglion [103–105], while a second type (i.e., egestion of a tube) is not [59]. As might be expected, therefore, rejection-like motor programs can be triggered in the isolated nervous system via CBI stimulation. Additionally, they can be triggered in the isolated buccal ganglion without the cerebral ganglion present [for a description of motor programs that would presumably be classified as egestive-like, see ref. 72]. Below, we discuss each type of motor program.

Without the Cerebral Ganglion (e.g. Stimulation of the Esophageal Nerve)

The buccal ganglion of *Aplysia* innervates the buccal mass via four bilaterally symmetrical nerves [55], which have been referred to as the esophageal nerve, buccal nerve 1, buccal nerve 2, and buccal nerve 3 [94; for other nomenclature, see ref. 106]. Additionally, a single radula nerve exits in the buccal ganglion from the region of the buccal commissure [94, 106]. In general, these nerves include afferent fibers [55]. Consequently, they can be used to trigger rhythmic activity [13, 28, 72].

Of the motor programs triggered via nerve stimulation, those triggered via the esophageal nerve are perhaps the best understood (at least in terms of their physiological significance). In particular, the esophageal nerve transmits mechanoafferent information from the gut of *Aplysia* to the buccal ganglion. As animals feed, information that is specifically conveyed via the anterior branch appears to be important for positive reinforcement of ingestive behaviors [26–28, 30, 31]. As feeding progresses, however,

the gut is stretched and animals begin to satiate [3, 14, 107]. When animals are satiated, they either fail to respond to food or they egest it, if it is in large pieces.

When the esophageal nerve is stimulated in semi-intact preparations, egestive movements are observed [13]. Similarly when the esophageal nerve is stimulated in the isolated nervous systems, egestive-like motor programs are observed. These motor programs are not as well characterized as programs triggered by CBI-2, but it has been noted that a number of the protraction and retraction neurons activated during CBI-2-elicited motor programs are also activated by esophageal nerve stimulation. For example, B4/5 are activated during retraction [97, 108], and B31/B32 are activated during protraction [72]. Programs are considered egestive-like since radula closer motor neurons predominately fire during the protraction phase of the motor program. As is the case with CBI-2-elicited motor programs, however, quantitative methods are often needed to classify many cycles of activity.

Rejection-Like Activity Triggered by Cerebral Neurons

As described above, rejection-like motor programs can be triggered by CBI-2 if CBI-3 is not recruited. Additionally, a CBI that appears to exclusively trigger rejection activity has been characterized, i.e. the neuron CBI-1. This cell has been identified and characterized in both *Aplysia californica* [60] and *A. kurodai* [105]. In *A. kurodai,* the designation CBM1 is used [105]. In *A. californica,* CBI-1 responds to touch of the tentacles, lips, and buccal mass [60]. In part, mechanoafferent input to CBI-1 is provided by the interganglionic cerebral to buccal mechanoafferents [60]. Tonic stimulation of CBI-1 generally produces a single cycle of a motor program that is characterized by high frequency activity in B20 during the protraction phase of the motor program and high frequency activity in B4/5 during the retraction phase of the motor program. As described above, this type of activity is generally considered rejection-like.

In *A. kurodai,* CBM1 is specifically associated with active rejection [105]. Thus, *A. kurodai* feed well on one type of seaweed, i.e. ulva, but they reject a second type, i.e. gelidium [104]. When gelidium is rejected, distinctive rhythmic patterned movements of the jaws and radula are observed [103]. For example, activity in jaw-closing motor neurons is phase advanced with respect to activity in MA neurons. (The MA neurons are presumably homologous to the B4/5 neurons in *A. californica.*) This type of change in rhythmic activity is observed when CBM1 is activated [105]. Additionally, imaging studies have indicated that gelidium extracts strongly excite CBM1 [105]. Interestingly, CBIM1 (and CBI-1) are dopaminergic [60, 105], as is B65 [109] and B20 [93], i.e. buccal interneurons that have also been associated with egestive-like activity [67, 110].

To summarize, *Aplysia* generate egestive as well as ingestive responses. These behaviors are interesting in that they do not always require the cerebral ganglion. In general, the egestive circuitry is not as well characterized as the ingestive circuitry but it appears that many of the same essential neurons are utilized. Dopaminergic neurons (e.g. CBI-1) have been particularly implicated as being important for generating egestive-like motor programs.

Concluding Remarks

The *Aplysia* feeding circuitry is organized in a manner that creates a great deal of potential for flexibility. For example:

(1) The initiation of behavior appears to be a complex process that does not simply involve the recruitment of a single command-like neuron. Instead, multiple cells are likely to be coactivated in a stimulus-dependent manner. The number of possibilities for behavior initiation is, therefore, not simply determined by the total number of command-like neurons. Instead, many different combinations of activity are possible.

(2) Once programs are ongoing, parametric features of motor programs are highly variable. In the context of protraction, this is likely to result from the following. (a) Protraction is in part sustained via a maintained depolarization in B31/B32. In part, this depolarization is induced by plateau-like potentials in B31/B32. Additionally, however, synaptic input is also important. Synaptic input to B31/B32 can be variable since interneurons not essential for protraction movements can be recruited in a behavior-specific manner. (b) Protraction duration is additionally affected by retraction initiation. Retraction initiation is itself under complex control. Thus, the retraction circuitry appears to be both inhibited and excited during protraction. Consequently, protraction duration can be altered either by a change in retraction phase inhibition, or a change in retraction phase excitation.

(3) Parametric features of retraction are also highly variable. In part, this is likely to result from the fact that some retraction interneurons are multifunction cells, i.e., they have peripheral processes and can function as primary afferents as well as interneurons. These neurons can therefore respond to a change in the environment and

alter the duration of the retraction phase. This can produce a change in the nature of an ongoing motor program (e.g. convert a biting-like program to a bite-swallow-like program).

(4) Finally, the essential nature of a motor program (i.e., whether it is ingestive-like or egestive-like) can be dynamically adjusted. At least in part, this is a result of the fact that a number of interneurons can exert both fast and slow synaptic actions. Consequently, a single cell can modify the two antagonistic phases of a motor program and thereby fundamentally change its nature (e.g. inhibit radula closer motor neurons during protraction and excite radula closer motor neurons during retraction, thereby making a program ingestive-like).

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Review

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Cephalopod Neural Networks

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Squid · Octopus · Statocyst · Vision · Chromatophore · Memory

Abstract
Cephalopods have arguably the largest and most complex nervous systems amongst the invertebrates; but despite the squid giant axon being one of the best studied nerve cells in neuroscience, and the availability of superb information on the morphology of some cephalopod brains, there is surprisingly little known about the operation of the neural networks that underlie the sophisticated range of behaviour these animals display. This review focuses on a few of the best studied neural networks: the giant fiber system, the chromatophore system, the statocyst system, the visual system and the learning and memory system, with a view to summarizing our current knowledge and stimulating new studies, particularly on the activities of identified central neurons, to provide a more complete understanding of networks within the cephalopod nervous system.

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Introduction

The coleoid cephalopods, comprising the squid, octopus and cuttlefish, have arguably the most advanced nervous systems amongst the invertebrates and certainly the most sophisticated systems within the phylum Mollusca [1–5, for reviews]. The only other extant group within the class Cephalopoda is the Nautiloids, which consist of 5 species that have retained the heavy external protective shell but have relatively simple nervous systems that presumably reflect the ancestral, more primitive form [6]. Although the nervous systems of the coleoid cephalopods are large and complex, containing up to 10^8 neurons [7], the primal organization of the nervous system, with distributed ganglia, can still be discerned in the large brain in that it is divided into distinct lobes connected by neural tracts and connectives. This can be clearly seen in sections of the squid brain (fig. 1), where the lobes of the dorsal part of the brain are partially separated from those in the ventral part of the brain by the oesophagus, which passes through the brain centre.

Although the anatomy of the cephalopod nervous system (CNS) has been well described for a few species, e.g. *Sepia officinalis* [8], *Octopus vulgaris* [9], *Loligo vulgaris* [10, 11], and the giant axons of the squid are perhaps the most intensively studied and modelled neurons in the whole of neuroscience, there is much less information available on the physiological activity and connections within the cephalopod CNS. This review focuses on a few of the systems where such information is available but the need for further investigations and more detailed information, particularly physiological activity data, will be apparent throughout.

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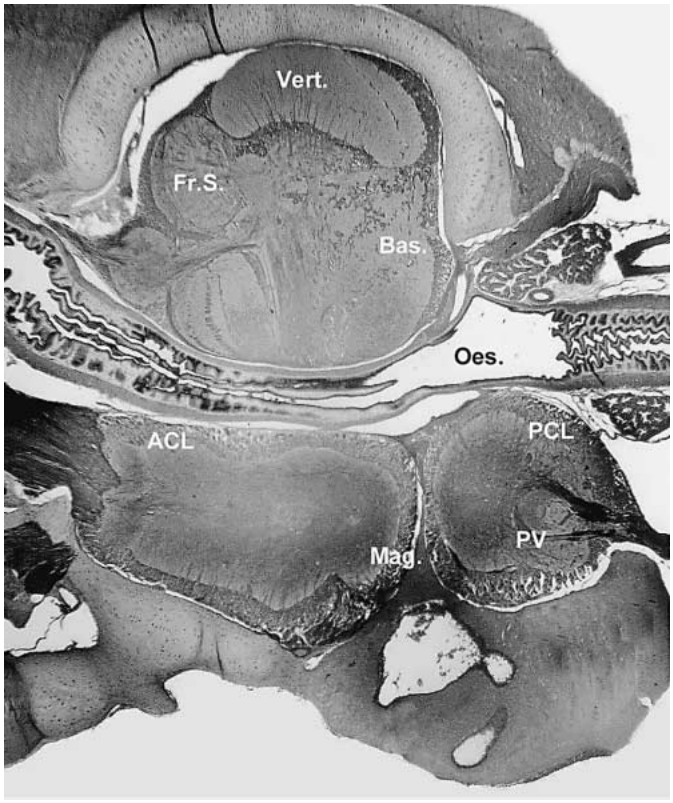

Fig. 1. Sagittal histological section through the brain of the squid, *Alloteuthis subulata*, showing some of the major lobes within the brain. Anterior chromatophore lobe (ACL), basal lobe (Bas.), superior frontal lobe (Fr.S.), magnocellular lobe (Mag.), oesophagus (Oes.), posterior chromatophore lobe (PCL), palliovisceral lobe (PV), and vertical lobe (Vert.).

The Giant Fiber Network

The giant fiber system is perhaps the most famous neural network system in cephalopods and comprises a chain of 3 interconnected giant nerve cells on each side of the animal. The system is present in most squid and cuttlefish, but rarely in octopuses, and is used in the jet-propelled escape response where sensory input, mainly from the eyes, results in a rapid contraction of specific muscles in the body mantle; this explosively expels water through the animal's funnel and produces a rapid backward escape movement of the animal. There are obvious parallels here with the C-start, escape response seen in some fish, driven through the Mauthner cell system [12], and the tail-flip escape response seen in some crustaceans [13].

The squid giant fiber system starts with the first order giant cell (soma up to 150 μm in diameter) lying within the ventral magnocellular lobe of the brain (fig. 1, 2). Information obtained from electrophysiological recordings from this cell [14], and from its morphology, position within the brain relative to other neuronal groups and the behavioural data showing how the system can be activated [15, 16], indicate that it receives major inputs from the visual and vestibular systems. Thus a perceived visual threat, or perhaps patterns of water borne vibrations detected by the statocyst or lateral line system, will provide sufficient excitation to trigger activity in the axon of the first-order giant fiber. The axon from this cell runs to the palliovisceral lobe where it crosses to the contralateral side and makes contact with its contralateral equivalent and then goes on to make synaptic contact with several second-order giant neurons (fig. 2) within the palliovisceral lobe [17, 18]. The contact between the two first-order giants is very strong and ranges from membrane fusion in the squid, to a large, presumed electrical synapse, in the cuttlefish. This arrangement ensures that both sides of the descending giant fiber system are activated simultaneously during any escape-type response. Some of the second-order giant fibers innervate the retractor muscles of the head and funnel but in squid a single large axon exits the brain in the pallial nerve and runs to the stellate ganglion in the mantle body, where it connects, via a 'giant synapse', to the third-order giant cells. The third-order giant cells innervate the mantle musculature via axons in the stellar nerves, the most posterior of which is generally known as 'the squid giant axon' and has been the subject of detailed physiological investigations since its description by Young [19, 17]. The third order giant fibers are syncytial in that each axon is supported by up to 100 cell somata [20] and the resulting giant axon can be up to 1,500 μm in diameter [21]. Direct electrical stimulation of the first-order giant cell, via an electrode in the magnocellular lobe [22], results in a short latency action potential in the pallial nerve (second-order cell active), followed by a spike in an ipsilateral stellar nerve (third-order cell active). The synaptic connection between the second- and third-order cells in the stellate ganglion is sometimes known as the squid giant synapse and its morphology [23], physiology and pharmacology [24, 25] have been well studied, particularly since it is feasible to place intracellular electrodes simultaneously in both the pre- and post-synaptic cells, as well as use imaging techniques to monitor changes in the concentrations of intracellular ionic species, such as calcium [26, 27]. Despite the concerted interest in this synapse, it is only recently that glutamate has been tentatively confirmed as the principal neurotransmitter, which perhaps serves as a useful reminder of how difficult it can be to firmly identify central

neurotransmitters in invertebrates. The results of much of the work on the giant synapse have recently been reviewed by Llinas [25] and clearly show the value and promise of this network as a model system for investigating the fundamental mechanisms involved in synaptic transmission.

The giant fiber system described here is of course a simplified version of the larger neural network that controls swim-jetting behaviour in squid and cuttlefish and even many of the escape-type responses often involve non-giant fiber activity that introduces more flexibility into the behaviour and may also act to compensate for fatigue when the system is repeatedly activated [16].

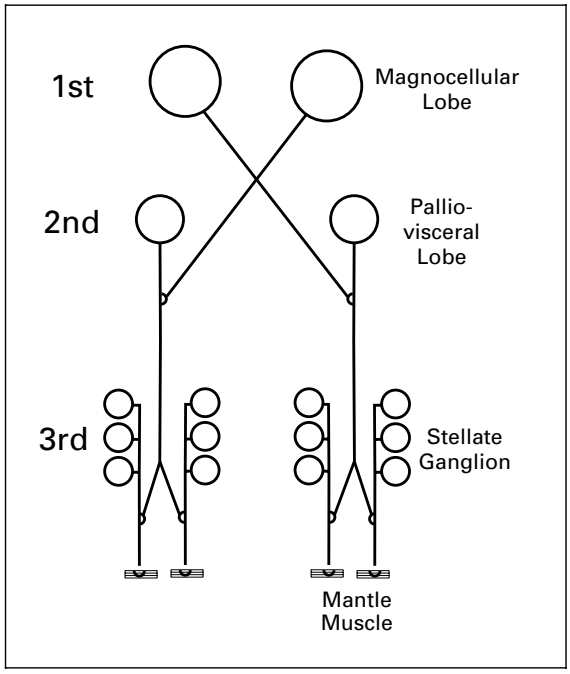

Fig. 2. Schematic representation of the giant fiber network in the squid. Right and left: first-order giant fibers have their cell bodies in the magnocellular lobe of the brain but, as their axons cross over to make synaptic contact with the second-order giant cells in the opposite side of the palliovisceral lobe, they fuse briefly together in a chiasma. The second-order giant cells send an axon out of the brain in the pallial nerve to make contact with the third-order giant neurons within the stellate ganglion. Note that the third-order giant cells are supported in a syncytial arrangement, with multiple cell bodies.

The Chromatophore System Control Network

Cephalopods can very rapidly change the colour and patterning of their skin through direct neural control of skin chromatophore organs [28]. They use these skin colour changes for both inter- and intra-specific signaling and for crypsis and camouflage [29]. The system is based upon thousands of elastic sacs embedded in the skin, each filled with a choice of coloured pigments [30] and each surrounded by a set of radial muscles which when active expand the sac to display the colour within, but when inactive permit the elastic sacs to contract to a small, near invisible, specks of colour. Thus, whole areas of the skin can change colour almost instantaneously, and static or active patterns can be displayed by activation and inactivation of groups of chromatophores. Note that this cephalopod skin colour system differs from those found in some fish, amphibians, reptiles or crustaceans in that it is not controlled through the endocrine system but directly controlled by muscles that are innervated from neurons whose soma are located in the brain.

The skin patterns produced by the cephalopod chromatophore system are strongly influenced by the visual environment of the animal; thus different signaling patterns can be produced in the presence of prey, predators, potential mates or rivals, as well as when matching the background or substrate with appropriate skin camouflage patterns [29]. The importance of the visual input seems to be reflected in the central nervous control system for it appears to be organized in a strictly hierarchical fashion (fig. 3), with the highest level of control located in the optic lobes, which are also largely concerned with processing the visual input from the eyes. From the optic lobes there are projections to the intermediate control centers in the lateral basal lobes (BL) of the brain, lying on the right and left posterior side of the supra-esophageal lobe, and from here there are large fiber tracts connecting to the anterior and posterior chromatophore lobes (ACL and PCL, respectively) where the majority of the chromatophore neuronal somata are located (fig. 3). There are numerous additional tracts and interconnections to other brain regions that make up this chromatophore control network, including projections back to the optic lobes [9] and peduncle lobes (Ped.) [28]. The importance and veracity of these descending pathways in the control network have been elucidated from anatomical tracings [9–11, 31–34], ablation experiments [35–38], electrophysiological recordings [39, 40], and focal electrical stimulation [31, 41, 42]. Thus, electrical stimulation of areas within the optic lobes has been shown to evoke complete and recognizable skin patterns, whereas stimulation of the lower motor centers containing either the chromatophore neuron somata, or the intermediate brain nuclei that innervate the ACL and PCL, evokes only localized skin

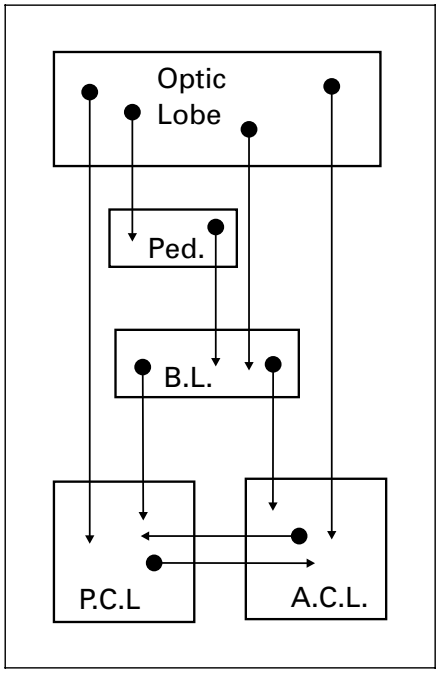

Fig. 3. Schematic representation showing the interconnections between the optic lobe, peduncle lobe (Ped.), lateral basal lobe (B.L.), and anterior and posterior chromatophore lobes (A.C.L. and P.C.L., respectively). The visual input from the eyes is processed within the optic lobes and this influences the selection of skin pattern that is then orchestrated through the peduncle and lateral basal lobes, before being activated from the motoneurons in the anterior and posterior chromatophore lobes.

colour changes or incomplete skin patterns. Although neurons within the ACL generally innervate chromatophores on the head and arms, whereas neurons in the PCL generally innervate chromatophores on the mantle, there is no clear evidence for a more detailed topographical matching of neuronal cell body location within the lobe and chromatophore location on the body [31, 43]. There is evidence however, from focal electrical stimulation of PCL motoneurons, that the chromatophores are organized into motor units of between 6 and 60 chromatophores and that individual chromatophore muscles are innervated by more than one motoneuron [44]; thus some basic skin patterns could be 'hard-wired' within the PCL or ACL and selected or mixed by the activation of specific motor units. However, more recent work [40] has shown that many PCL neurons are dye-coupled, a common indication of electrical coupling, and this could undermine the finding of Dubas et al. [43]. Finally, it should be noted that there is no known feedback from the chromatophore units to the CNS, other than through visual monitoring, and of course, not all chromatophores are visible to the animal itself. This, therefore, is an example of a complex and dynamic motor output that appears to have a limited or incomplete feedback system.

The Statocyst Network

The cephalopod statocysts are the principal sense organs detecting body orientation with respect to gravity and movements in space; they operate and perform in a manner, and at a level of sophistication, that is similar to that of the vertebrate vestibular system [45–48]. The coleoid cephalopods have two bilaterally symmetric statocysts located just ventral to the brain, and embedded in the cranial cartilage. Each of these right and left statocysts has two separate receptor systems: one which detects the linear accelerations including gravity, the macula/statoliths system, and the other which detects angular accelerations, the crista/cupula system. A significant body of morphological and electrophysiological data has been obtained from the crista/cupula and macula/statolith systems showing how they are constructed, their response characteristics and the network of interconnections that modulates and controls their operation. The angular acceleration receptor system consists of thin strips (cristae) of sensory hair cells and associated neurons which run around the inside of the statocyst approximately in the three orthogonal planes of the animal. In decapods such as squid and cuttlefish, the crista is subdivided into four segments, while in octopus, into nine segments. Each one of these crista segments (fig. 4a) carries an overlying gelatinous cupula which is attached to the tips of the mechanosensory hair cells and moves like a sail as the endolymph fluid within the statocyst cavity shifts during a head movement and thus stimulates the underlying sensory hair cells [47–49]. The sensory epithelia of the crista segments (fig. 4a) contain three main cellular elements: (1) sensory hair cells arranged in up to eight rows, and subdivided into two main types: the primary hair cells (with an axon passing towards the brain) and large and small secondary hair cells (without an axon but making synaptic contact with afferent neurons); (2) first-order afferent neurons lying close to the sensory hair cells and also comprising two sub-types, i.e. large and small primary afferent neurons, and finally (3) efferent cell inputs from the brain that innervate the sensory hair cells as well as the first-order afferent neurons [50]. Electrophysiological recordings from the statocyst have shown that the macula/statolith system responds to gravity but also to

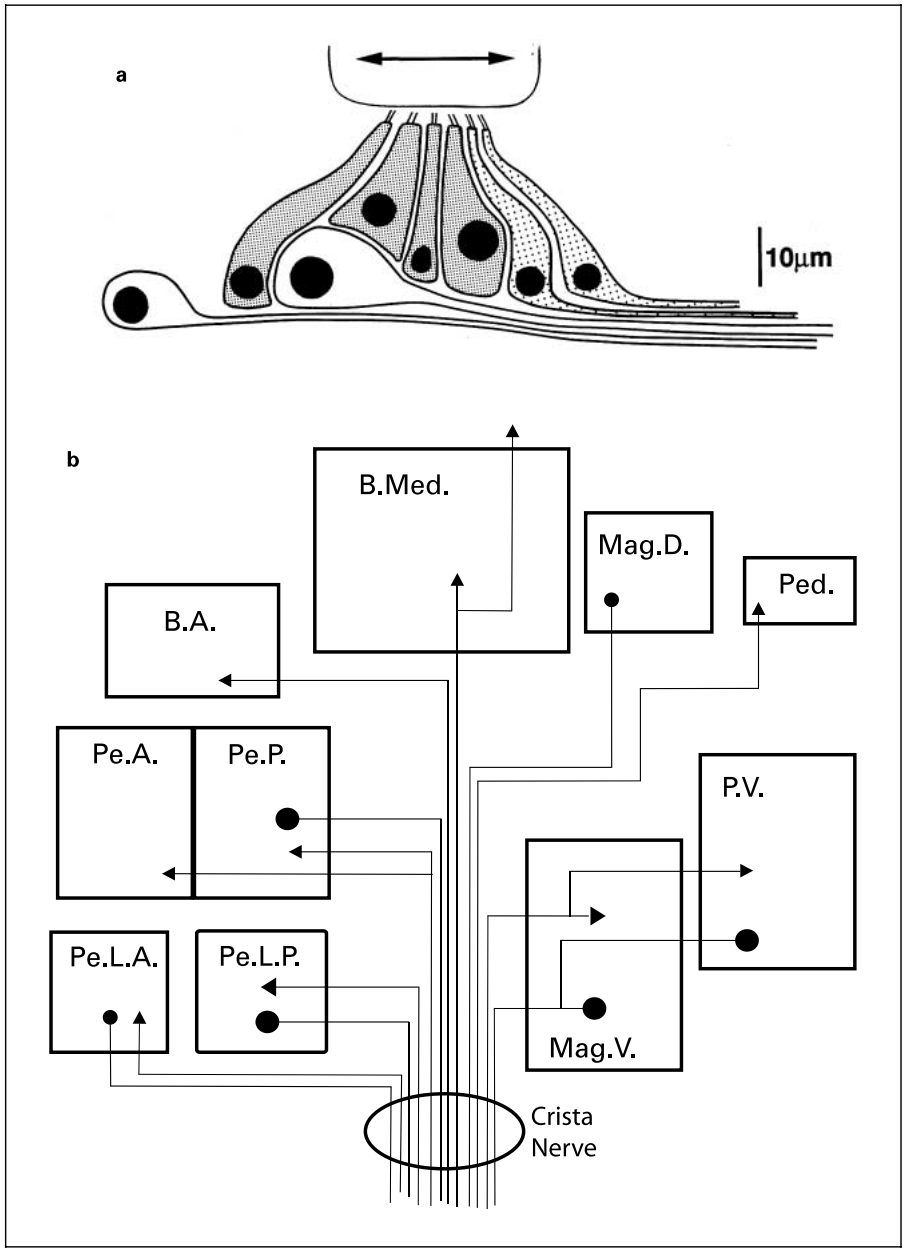

Fig. 4. a Schematic representation of a cross-section through the crista epithelium of the squid statocyst showing the overlying cupula, the secondary mechanosensory hair cells (dark stippled), the primary mechanosensory hair cells (light stippled), and the large and small afferent neurons (unstippled). The large efferent input to the system is not shown. **b** Block diagram showing the main output and input connections between the statocyst and brain in Octopus. The connections were revealed by dye tracings of the Octopus posterior crista nerve [after 67]. The arrowheads indicate afferent projections from the statocyst, whereas the filled circles indicate the sites of efferent cell bodies. Anterior basal lobe (B.A.), median basal lobe (B.Med.), dorsal magnocellular lobe (Mag.D.), ventral magnocellular lobe (Mag.V.), peduncle lobe (Ped.), anterior pedal lobe (Pe.A.), posterior pedal lobe (Pe.P.), anterior lateral pedal lobe (Pe.L.A.), posterior lateral pedal lobe (Pe.L.P.) and palliovisceral lobe (P.V.).

vibrations in the surrounding fluid [51] whereas the crista/cupula system acts as an angular velocity detector and has functional response characteristics similar to those of the vertebrate semicircular canal system [52]. The cellular elements within the crista epithelia, and presumably also within the sensory epithelia of the maculae, are linked by a network of synaptic interconnections and receive a very powerful efferent input from the brain; both of these operate to modulate and control the afferent information to the brain.

Synaptic Interaction between Mechanosensory Hair Cells

Paired intracellular recording from neighboring primary sensory cells revealed that action potentials in one primary hair cell resulted in a one-for-one transmission to the other cell and this appeared either as a post-synaptic potential or as an action potential [53]. Furthermore, injection of depolarizing or hyperpolarizing currents into a primary hair cell resulted in a depolarization or hyperpolarization of the respective neighbour, demonstrating

that they are electrically coupled and with coupling coefficients of ≤ 0.4 for the primary sensory hair cells [53, 54]. The secondary sensory hair cells, which are located on the outermost ventral side of the horizontal crista segments, were also shown to be coupled electrically with higher coupling coefficients of ≤ 0.6 [55]. There was no electrical coupling found between the primary and secondary, but this was expected as these hair cells are known to be functionally polarized in the opposite directions [49, 56, 57] and so such coupling would act to cancel out their responses. The advantage of electrical coupling between the sensory hair cells may be that it acts to improve the signal to noise ratio of the system, at the expense of reducing the high-frequency response, and thus improves the overall sensitivity.

Efferent System

The statocyst efferent cells make up a major proportion of the axons within the statocyst nerves with, on average, 75% of the axons considered to be efferent fibers [50, 57]. Individual sensory hair cells and first-order afferent neurons receive more than 15–30 efferent endings, respectively [50, 59] and these efferent inputs can have very powerful effects on the afferent responses. Recordings from crista cells have shown that the majority of the efferent innervation onto the hair cells and the first-order neurons is inhibitory but excitatory and mixed effects are also present [53, 56]. Although electrical, efferent synaptic connections cannot be ruled out, many of the efferent contacts onto the sensory hair cells or the afferent neurons have been shown to be chemically mediated [53, 54] with morphological and histochemical evidence for the presence of two types of efferent populations with two different neurotransmitters [50, 61, 62]. By direct electrical stimulation of the efferents [63, 64], while recording the afferent output from the statocyst [51], or by pharmacological application of acetylcholine to mimic inhibitory efferent action and catecholamines to mimic excitatory efferent actions [60], it has been shown that the efferents have very powerful, but selective actions on the statocyst sensory epithelium. A further complication to the control and activity within this network is that there is evidence that the strength of the electrical coupling between the hair cells and afferent neurons may be modulated by the efferent innervation [65, unpublished data].

Interactions between Hair Cells and First-Order Afferent Neurons

Using light- and electron-microscopic studies of *Octopus vulgaris*, Budelmann et al. [57] demonstrated that there are two types of first-order afferent neurons, those with large somata (diameters between 20 and 35 μm) and those with small somata (diameters 5–15 μm). They also demonstrated that the large afferent neurons probably receive much of their input from the large second-order mechanosensory hair cells while the smaller afferent neurons receive their input from the smaller second-order hair cells. However, there is a clear difference in the relationships between these two pathways in that there are roughly 4 large hair cells to each large afferent neuron, indicating a convergence of information flow, whereas there are roughly twice as many small afferent neurons compared to the number of small second-order hair cells, indicating a divergence of flow. Although the synaptic connections between the second-order sensory hair cells and the first-order neurons appear chemical, on the basis of ultrastructural data and some physiological evidence [46, 56, 66], recent results have indicated that there may also be some electrical coupling between these cell groups [53].

Central and Efferent Projection of the Statocyst Nerves

Dye fills of the nerves from the statocysts [67] in octopus show that the axons from the primary afferent neurons and primary sensory hair cells from the cristae and maculae epithelia project directly and indirectly to numerous centers within the brain (fig. 4b) with the main areas being (1) the ipsilateral, and lateral parts of the contralateral, anterior pedal lobes, (2) the ipsilateral, and the contralateral, posterior pedal lobes, and (3) the ventral brachial and ventral magnocellular lobes. Further dye injections into the octopus brain [67] showed that the perikarya of the maculae and cristae efferents were located dorsally and ventrally in the lateral parts of the anterior palliovisceral lobe and posterolaterally in the posterior pedal lobes (fig. 4b). Some perikarya were also seen in the anterior lateral pedal lobe.

Thus the cephalopod statocyst is a sophisticated sense organ that rivals the vertebrate vestibular system in its functional characteristics and contains an intricate network of neural connections that acts to modulate and control its operation and output. In terms of investigating how such complex networks operate, the cephalopod vestibular system offers significant advantages for the experimenter over the analogous vertebrate system in that it is accessible, embedded in soft cartilage instead of bone, the afferent neurons have their somata in the periphery and hence paired recordings can be made from hair cells and their afferents, and there is a very large efferent innervation (70% of the fibers in the nerve compared with 8–18% in vertebrates) with powerful and diverse effects.

The Visual System

Cephalopods are highly visual predators utilizing a pair of large, elaborate eyes that are often cited as a textbook case of convergent evolution because of their close parallels with vertebrate eyes [2]. Both groups have single chamber, camera type eyes, with focusing lenses, variable sized pupils, large retina with foveal areas where the receptor cells are more densely packed to increase acuity, and a screening pigment that migrates outwards under high light intensities [7, 68–71]. There are nevertheless distinct differences in the cephalopod system in that: (1) the photoreceptors are of the invertebrate, rhabdomeric type [70], with each photoreceptor having an axon that exits at the back of the retina and so there is no blind spot in the cephalopod retina; (2) only rod-like photoreceptors are present in the cephalopod retina and these appear sensitive to a narrow range of light wavelengths and so vision is almost exclusively monochromatic [but see 72]; (3) polarized vision is common in cephalopods [73, 74]; (4) the retina contains only the photoreceptors and the terminals of an efferent projection and so visual processing, as found in the vertebrate retina, is most likely relocated to the optic lobe region of the brain [75, 76].

The cephalopod optic lobes are large CNS areas, lying just behind the eyes, which receive the photoreceptor axons from the eyes and are connected to the rest of the brain and motor centers through large optic tracts. It is within these optic lobes that the major processing of visual information is believed to occur [e.g. 9, 77–79]. Each of the paired optic lobes has two anatomically distinct areas; an outer cortex and a central medulla [e.g. 9, 31, 80, 81]. The outer cortex, also called the 'retina profunda' or 'deep retina' [8] due to its similarity with the ganglionic layer of the vertebrate retina, is where most of the processing and classification of the visual inputs from the retina is thought to occur [81]. This cortex is mainly composed of two layers of cell somata; the outer and inner granule cell layers that are separated by a complex neuropil zone, the plexiform zone [10, 31]. In octopus, the outer granule layer comprises mainly amacrine neurons lacking axons, but in decapods, cells with axons running towards the medulla have also been reported [10, 82]. By contrast, the inner granule cell layer in both octopods and decapods has a more varied cellular composition, containing four main cell types: reverse amacrines and centrifugal cells as well as the more centrally occurring centripetal and multipolar neurons [9, 10, 81, 82].

The much larger central medulla area is considered as a visuomotor region which also serves as a memory centre [45, 82]. The medulla consists of numerous clusters of cell bodies (or cell islands) separated by tracts of fibers (neuropil), containing both axons and dendrites. The cell islands contain many large unipolar cells that branch into numerous, presumably dendritic, branches, one of which forms an axon which passes towards the optic tract. This tract provides the communication between the optic lobe and other areas of the CNS [9, 10, 83].

Synaptic Interactions within the Visual System of Cephalopods

Although the cephalopod retina contains only photoreceptors and supporting cells, the photoreceptors have basal processes that could permit interactions between photoreceptors [75, 76]. This view is supported by single unit, extracellular recordings from the photoreceptor axons that indicate that the retinal outputs are already organized into 'on-off' receptive fields [84], presumably through a network of direct photoreceptor to photoreceptor interactions. In addition, the efferent input to the retina from the brain has been shown to modify the retinal responses, again presumably through synaptic connections with the photoreceptors within the retina [76].

Within the optic lobe, the retinal photoreceptors terminate mainly in the plexiform zone of the outer cortex [9, 10] and are retinotopically mapped onto the lobe [85]. Morphological studies indicate that the photoreceptors make synaptic contact with the amacrine neurons located in the outer and inner granule cell layers [86] and this is supported by field potential recordings showing synaptic responses within the plexiform zone 2–6 ms after optic nerve stimulation [87]. The neurotransmitter employed by the photoreceptors is likely to be acetylcholine [88, 89]. Using a brain slice preparation, Chrachri and Williamson [90] have recently shown that stimulation of an optic nerve bundle in cuttlefish evokes excitatory postsynaptic currents in amacrine neurons located in the inner granule cell layer, supporting the view that these neurons are in direct synaptic contact with the retinal photoreceptors. A class of centripetally running neurons, with somata in the inner granule cell layer, has their dendritic fields organized in specific planes and directions within the plexiform zone such that they could extract orientation information from the visual field [9, 10, 81]. The axons of these centripetal neurons pass into the medulla and physiological recordings of their activity [91] support the view that these are second order visual neurons. Within the medulla, Young [9] identified radial columns of cells that make lateral interactions as they proceed deeper into the medulla and he proposed that these make contact with feature-

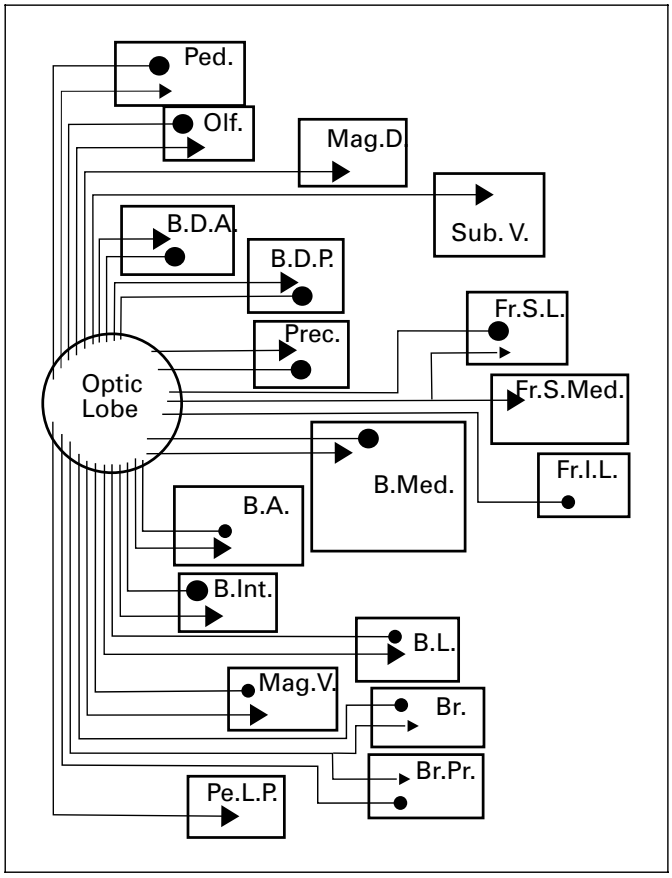

Fig. 5. Block diagram showing the main output and input connections between the optic lobes and other brain regions in Octopus. The connections were revealed by dye injections and tracings from the optic lobes [after 67, 83]. The arrowheads indicate afferent projections from the optic lobe, whereas the filled circles indicate the sites of efferent cell bodies. Anterior basal lobe (B.A.), anterior dorsal basal lobe (B.D.A.), posterior dorsal basal lobe (B.D.P.), interbasal lobe (B.Int.), lateral basal lobe (B.L.), median basal lobe (B.Med.), brachial lobe (Br.), prebrachial lobe (Br.Pr.), lateral inferior frontal lobe (Fr.I.L.), lateral superior frontal lobe (Fr.S.L.), median superior frontal lobe (Fr.S.Med.), dorsal magnocellular lobe (Mag.D.), ventral magnocellular lobe (Mag.V.), olfactory lobe (Olf.), peduncle lobe (Ped.), posterior lateral pedal lobe (Pe.L.P.), precommissural lobe (Prec.) and subvertical lobe (Sub.V).

detecting neurons. As yet there is no physiological evidence to support this hypothesis although Chrachri and Williamson [unpubl.] have recorded from neurons within the medulla that responded with volleys of EPSCs to stimulation of the optic nerve photoreceptor axons.

The efferent neurons that innervate the retina have their somata in the inner granule cell layer of the optic lobe and could modify the photoreceptor responses through action on the cell membrane potentials, the electrical coupling between cells, and through changes in the screening pigment location [71, 92]. Intracellular recordings from these efferent neurons show that they receive short latency inputs immediately after stimulation of the photoreceptor axon bundles, implying that there are direct connections between the afferent and efferent networks [93; Chrachri and Williamson, unpubl.].

Central Connections of the Optic Lobes

The output and input pathways between the optic lobes and the rest of the brain have been studied by a variety of histological and dye tracing techniques [9, 67, 83] and a large number of both direct and indirect projections were identified. A summary of the direct, ipsilateral pathways from the octopus optic lobe to the central brain is shown in figure 5. In the main, the optic lobe output fibers project to various divisions of the prebrachial, brachial, pedal, magnocellular, basal, subvertical, peduncle, olfactory and contralateral optic lobes. Additional smaller projections have also been observed, as well as numerous possible indirect projections. However, without supporting evidence, such as physiological recording or stimulation data, these are difficult to interpret. Inputs to the optic lobes from the central brain arise from many of the main output areas identified above (fig. 5) as well as from the frontal and inferior frontal lobes. The peduncle and olfactory lobes located on the optic tract [94], the former sometimes known as the cephalopod cerebellum [95], seem particularly well situated to play a major role in the processing of visual information. Similarly, the magnocellular lobe, which is involved in the escape behaviour, and the vertical lobe, which has been shown to be involved with both visual and tactile memory [9], both appear strong candidates for further physiological studies of visual evoked activity.

Networks Involved in Learning and Memory

Learning and memory capabilities are well developed in cephalopods and there is a substantial body of work describing their performance in various forms of short- and long-term memory tasks [e.g. 28, 96–99] as well as in tests of habituation, conditioning, associative learning, discrimination learning, and even social learning [e.g. 9, 15, 100–103]. These reports show that cephalopods can rival the accomplishments of many vertebrates in such tasks [e.g. 104]. However, in experiments testing sensory discrimination, where partial brain ablations were also performed, it was found that there appear to be two quite

distinct central memory systems present, one for visual tasks and another for tactile/taste tasks [105–107] and that: (1) tactile learning is mostly associated with the sub-frontal lobes; (2) visual learning is associated with the optic lobes; (3) damage to the vertical and sub-frontal lobes does not affect movement nor posture, but is specifically disruptive to learning, and finally (4) lesions to the vertical lobe affect both visual and tactile learning systems [for reviews, see 9, 11, 32, 108, 109].

The Visual Memory System

The store for visual memory appears to be associated with the optic lobes, but there is an important ancillary circuit involving the median superior frontal lobes and the vertical lobe [99, 110–113]. Experimental evidence indicates that visual memories are laid down bilaterally, although normally an octopus attacks with the prey image in the visual field of a single eye, implying that the memory established in one optic lobe must be transferred to the contralateral optic lobe via the central, large commissure. Interruption of this commissure prior to training abolishes transfer while section afterwards does not [114].

The discrimination of the visual image is thought to take place in the optic lobe cortex via the pathway described above; i.e. the photoreceptor cells synapse with second-order neurons in the cortex of the optic lobe [9] and these second-order neurons (the amacrine and centripetal neurons) have dendritic fields [10] arranged to best extract particular aspects of the visual input (such as object orientation) and match this information with respect to body orientation, as given by the gravity detecting system in the statocysts. Axons from the second order neurons pass to the central medulla of the optic lobe where they combine with the inputs from other neurons in the visual pathway to form 'classifying cells'; this information is then transferred to other CNS areas where an appropriate response to the visual cue is elicited [99]. It has been postulated that visual inputs related to a potential predator, and thus likely to evoke an escape response, are communicated via identified pathways to the magnocellular lobes, for these are involved in direct motor responses, whereas visual inputs related to potential prey items, and thus likely to evoke an attack sequence, are communicated via separate pathways to the peduncle and basal lobes, which are involved in orchestrating more complex motor sequences [99].

Numerous other experiments have confirmed that visual discrimination tasks are impaired by lesions in the vertical lobe system [113, 115] and that if part of this lobe is removed, then the accuracy of the memory is proportionately reduced. Similar results have also been obtained after manipulations of the optic lobes [111]; however, animals without a peduncle lobe are still capable of learning visual discrimination tasks, but the execution of the responses is impaired [94]. Impairment of visual discrimination tasks can also be achieved through biochemical disruption for Robertson et al. [116] have shown that inhibition of the nitric-oxide synthase system blocks visual learning.

The Tactile Memory System

Cephalopods possess a separate memory system for touch learning [117]. This system takes information from the arms and numerous sensory receptors associated with the arm suckers [e.g. 118] and ensures that the arms draw food objects towards the mouth and reject non-food objects [119]. A clear example of this second system is the ability to learn to discriminate between rough and smooth objects; this is not a visual task, for the test objects used in these experiments could not be visually discriminated, and the task could be learned in the absence of the optic lobes (a necessary lobe for visual task learning), but could not be learned in the absence of the sub-frontal lobes [117]. Although this sub-frontal lobe contains the major tactile memory, the vertical lobe has also been shown to be involved in tactile learning [99, 117, 120]. As with the visual system, the brain systems involved in tactile learning can be disrupted by the administration of blockers of protein synthesis or nitric oxide synthase activity [116, 121].

Since the vertical lobe participates in both types of learning, it would be of considerable interest to see whether the same populations of neurons are involved in both, or whether there are separate pools for each. Physiological experiments in progress indicate that the octopus vertical lobe can exhibit long-term potentiation (LTP), a phenomenon closely associated with vertebrate memory, and it may be that the early analogies drawn between the vertebrate hippocampus and the cephalopod vertical lobe on the basis of structural similarities [e.g. 95] can be confirmed by physiological and behavioural experiments.

With regard to the neurotransmitters implicated in learning and memory, there is already substantial evidence for the involvement of cholinergic system in both vertebrates [122–124] and invertebrates [125–127]. Similarly, within the cephalopods, there is biochemical and pharmacological information on the distribution of acetylcholine within the CNS [128, 129] and clear evidence that disruption of the cholinergic systems in the higher CNS centers, such as in the vertical and frontal lobes,

interferes with both learned behaviours [130] and memory recall [131]. There is of course extensive evidence for the presence of almost all of the other major neurotransmitters and neuromodulators within the cephalopods CNS [for reviews see 31, 128], but apart from acetylcholine and nitric oxide there is little published work linking these specifically to memory and learning.

Conclusion

This short review of neural networks within the cephalopods has focused on just a few of the systems where there is complementary morphological, physiological and behavioural data that provide a basis for understanding the function and operation of the networks. There are a whole variety of other cephalopod systems, such as the control of the arms [132], the olfactory system [133], the oculomotor system [134], where there is already a considerable body of knowledge that will soon enable us to establish more completely how these systems operate and integrate into the overall behaviour of the animal. The cephalopods are a fascinating group of animals, capable of producing a wide repertoire of behaviour and, as this review has indicated, they provide excellent invertebrate models for the goal of understanding behaviour in terms of the operations of the underlying neural substrate.

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